

**NUCLEIC ACID MOLECULES AND POLYPEPTIDES
FOR A HUMAN CATION CHANNEL POLYPEPTIDE**

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This application claims benefit to provisional application U.S. Serial No. 60/257,865, filed December 21, 2000.

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1. FIELD OF THE INVENTION

The present invention relates to the isolation and identification of human nucleic acid molecules and proteins and polypeptides encoded by such nucleic acid molecules, or degenerate variants thereof, encoding a human cyclic nucleotide gated (CNG) cation channel. The proteins and polypeptides of the invention represent a novel cation channel that may be a therapeutically valuable target for drug delivery in the treatment of human diseases that involve calcium, sodium, potassium or other ionic homeostatic dysfunction, such as central nervous system (CNS) disorders, e.g., stroke, anxiety and depression, or degenerative neurological disorders such as Alzheimer's disease or Parkinson's disease, or other disorders such as cardiac disorders, e.g., arrhythmia, diabetes, chronic pain, hypercalcemia, hypocalcemia, hypercalciuria, hypocalciuria, or ion disorders associated with immunological disorders, gastro-intestinal (GI) tract disorders, or renal or liver disease. Moreover, the polypeptides of the present invention can function as effector molecules, reflecting the intracellular concentration of cAMP and/or cGMP. Accordingly the present invention also relates to the use of the CNG cation channel polypeptides disclosed herein for the detection of modulators of intracellular cAMP and/or cGMP levels. More specifically, the present invention relates to the use of CNG cation channel



polypeptides as components of assays for the detection of
5 antagonists and/or agonists of G-protein coupled receptor
activity, which may be therapeutically useful molecules.

2. BACKGROUND OF THE INVENTION

Control of the internal ionic environment is an
10 extremely important function of all living cells. Ion
exchange with the external medium is regulated by a
variety of means, the most important of which are various
transporters and ion channels. Ion channels in particular
have been important targets for the development of
15 therapeutic compounds in the treatment of disease.

A number of proteins have been described as forming
ion channels. Among these are proteins that have been
shown to function as cation channels of varying degrees
of selectivity and with different, and in some cases
20 unknown, mechanisms for channel gating. Within the family
of cation channels, there is an identified group that
includes cyclic nucleotide gated (CNG) channels, which
are activated by intracellular binding of cAMP and/or
cGMP to CNG polypeptides. CNG channels are nonselective
25 cation channels which allow the passage of monovalent
cations, including both K^+ and Na^+ ions, as well as
divalent cations. Although CNG channels can transport
both monovalent and divalent cations, Ca^{+2} blocks the flow
of monovalent cations through the channel (Zagotta et al.
30 1996 Ann. Rev. Neurosci. 19: 235-63). CNG channels were
originally found to be involved in signal transduction
within sensory tissues.

The first cDNA clone encoding a CNG channel
 α -subunit polypeptide was isolated from bovine rod tissue
35 (Kaupp et al. 1989 Nature 342: 762-66). Subsequently, a
series of CNG α -subunit polypeptide encoding genes were
isolated from other tissues and species that encoded

- proteins structurally related to the bovine rod CNG
- 5 α -subunit polypeptide. (Bauman et al. 1994 EMBO J 13:5040-50; Biel et al. 1993 FEBS Lett 329: 134-38; Biel et al. 1994 Proc. Natl. Acad. Sci. USA 91:3505-09; Bönigk et al. 1993 Neuron 10: 865-77; Bradley et al. 1994 Proc. Natl. Acad. Sci. USA 91: 8890-94; Chen et al. 1993 Nature
 - 10 362: 764-67; Dhallan et al. 1990 Nature 347: 184-87; Dhallan et al. 1992 J. Neurosci. 12:3248-56; Goulding et al. 1992 Neuron 8: 45-58; Liman et al. 1994 Neuron 13: 611-21; Ludwig et al. 1990 FEBS Lett. 270: 24-29; Weyland et al. 1994 Nature 368: 859-63). Although these genes
 - 15 were shown to be structurally related, different tissue-specific and species-specific expression of those genes was established (Distler et al. 1994 Neuropharmacology 33: 1275-82). For example, the full-length cDNA encoding the CNG channel polypeptide
 - 20 isolated from rabbit aorta was reported to be 93.7% homologous with bovine olfactory CNG polypeptide (Biel et al. 1993 FEBS Lett 329: 134-38). The functional role of the murine olfactory CNG polypeptide was established, *in vivo*, by constructing knockout mice lacking this gene. In
 - 25 these mutant mice, electrophysiological assays demonstrated that excitatory responses to odorants were undetectable, providing direct evidence for the role of this CNG channel in excitatory olfactory signal transduction (Brunet et al. 1996 Neuron 17: 682-93).
 - 30 A second, distinct cDNA clone encoding a CNG channel α -subunit polypeptide was isolated initially from olfactory tissue (Dhallan et al. 1990 Nature 347: 184-87; Goulding et al. 1992 Neuron 8: 45-58; Ludwig et al. 1990 FEBS Lett. 270: 24-29) and later from rabbit aortic
 - 35 tissue (Biel et al. 1993 FEBS Lett. 329:134-38).

A third distinguishable cDNA clone encoding a CNG channel α -subunit polypeptide has also been cloned from

both sensory and non-sensory tissues: cone photoreceptors
 5 (Bönigk et al. 1993 Neuron 10: 865-77), testis (Weyland
 et al. 1994 Nature 368: 859-63), and kidney tissue (Biel
 et al. 1994 Proc. Natl. Acad. Sci. 91: 3505-09).

Amino acid sequence comparisons between and among
 the encoded CNG α -subunit polypeptides identified above,
 10 as well observed regions of homology between these
 proteins and other ion channels polypeptides, have been
 used to construct a structural model for CNG α -subunit
 proteins (Zagotta et al. 1996 Ann. Rev. Neurosci. 19:
 235-63). In this model, both the N-terminal and
 15 C-terminal sequences of CNG α -subunit polypeptide are
 positioned within the cell, and the termini of the
 α -subunit protein are separated by six transmembrane
 segments, designated S1 to S6 when viewed in the
 N-terminal to C-terminal direction. The peptide segment
 20 spanning the region between S5 and S6 constitutes the
 surface of the pore through which cations are conducted.
 In addition, binding sites for Ca^{+2} -Calmodulin and cAMP
 and/or cGMP have been identified on the intracellular
 N-terminal and C-terminal peptide segments, respectively.
 25 Heterologous expression of the above α -subunit
 polypeptide encoding CNG genes alone in, for example,
 Xenopus oocytes, provides a functional ion channel.

Clones have also been isolated that encode a second
 polypeptide subunit, referred to as the β -subunit
 30 polypeptide, of CNG channels (Chen et al. 1993; Bradley
 et al. 1994; Liman et al. 1994). Hydropathicity analyses
 of the two identified β -subunit polypeptides and amino
 acid sequence comparisons indicate that the β -subunit
 polypeptides, like the α -subunit polypeptides, consist of
 35 cytoplasmic amino- and carboxyl-termini separated by six
 transmembrane segments, a binding site for cyclic
 nucleotides within the C-terminal, intracellular portion

of the protein, and an ion-conducting pore. Despite these structural similarities, there is only about a 40% amino acid sequence identity observed between the CNG α -subunit and β -subunit polypeptides, in contrast to the approximately 65% amino acid identity observed between the various CNG α -subunit polypeptide sequences.

Furthermore, and in contrast to the results obtained with the α -subunit CNG polypeptide, heterologous expression of the β -subunit CNG polypeptide alone does not provide a functional ion channel. However, co-expression of both α and β CNG subunits yields heteromeric complexes having properties exhibited by naturally-occurring CNG channels that are not observed with homomeric CNG complexes formed with the α -subunit alone, including an increased affinity for cyclic-nucleotide binding. The β -subunit CNG polypeptides have, therefore, been referred to as modulatory subunits of CNG channels (Biel et al. 1999, Reviews of Physiology Biochemistry and Pharmacology 135: 151-71). Therefore, CNG channels consist of complexes of homologous but distinguishable α -subunits and β -subunits.

Kinetic models have been proposed which correlate cyclic nucleotide binding with CNG channel opening. In one model, summarized by Zagotta et al. (Zagotta et al. 1996 Ann. Rev. Neurosci. 19: 235-63), addition of cyclic nucleotides to four cooperative binding sites induces allosteric, conformational changes which result in the opening of the CNG channel. The existence of multiple, cooperative cyclic nucleotide binding sites forms the basis of the exquisite sensitivity of CNG channels to variations in the intracellular concentration of cAMP and/or cGMP.

Cyclic nucleotides serve as intracellular second messengers involved in regulated gene expression in response to extracellular signals. Such signals may be

initiated, for example, by ligand binding to a G-protein
5 coupled receptor, inducing conformational changes leading
to intracellular activation of adenylyl or guanylyl cyclase.
Resulting increases in the concentration of cyclic
nucleotides can activate and open CNG channels, providing
an influx of monovalent and/or divalent cations, and
10 particularly calcium ions which, in turn, are directly
involved in many aspects of biochemical and genetic
regulation. It is through this biochemical cascade that
CNG channels function as effector molecules for
intracellular signals generated, for example, by
15 G-protein coupled receptors.

Therefore, CNG channels are critical mediators of
the cyclic nucleotide response generated in signal
transduction pathways. The distribution of CNG channels
within olfactory, auditory, brain, testicular, kidney,
20 cardiac, and central nervous system tissues, demonstrates
that CNG channels are important components of many
critical biological processes. As such, human CNG
channels are important targets, per se, for therapeutic
intervention. Furthermore, CNG channels are also useful
25 tools, in their role as effector molecules, for
reflecting the modulation of intracellular cyclic
nucleotide levels. Accordingly, CNG channels may also be
used in assay procedures and screening methods for
detection of compounds that modulate processes,
30 including, but not limited to ligand binding and signal
generation by G-protein coupled receptors, that affect
intracellular cyclic nucleotide levels.

5 3. SUMMARY OF THE INVENTION

The present invention relates to the isolation and identification of nucleic acid molecules and proteins and polypeptides encoded by such nucleic acid molecules, or degenerate variants thereof, that participate in the formation or function of human ion channels. More specifically, the nucleic acid molecules of the invention include a novel human gene that encodes a protein or polypeptide involved in the formation or function of a novel cation channel.

15 According to one embodiment of the invention, a novel, complete human cDNA, termed HBMYCNG, and the amino acid sequence of its derived expressed protein, is disclosed.

The compositions of this invention include nucleic acid molecules, e.g., the HBMYCNG gene, including recombinant DNA molecules, cloned genes or degenerate variants thereof, especially naturally occurring variants, which encode the HBMYCNG gene product, and antibodies directed against that gene product or conserved variants or fragments thereof.

In particular, the compositions of the present invention include nucleic acid molecules (also referred to herein as "HBMYCNG nucleic acid molecules" or "HBMYCNG nucleic acids") which comprise the following sequences:

30 (a) nucleic acid sequences of the human HBMYCNG gene, e.g., as depicted in FIG. 1, and as deposited with the American Type Culture Collection (ATCC) as disclosed in Section 7, *infra*, as well as allelic variants and homologs thereof; (b) nucleic acid sequences that encode the HBMYCNG, gene product amino acid sequences, as depicted in FIG. 2; (c) nucleic acid sequences of a variant of the human HBMYCNG gene, e.g., as depicted in

FIG. 5, and as deposited with the American Type Culture
5 Collection (ATCC) as disclosed in Section 7, *infra*, as
well as allelic variants and homologs thereof; (d)
nucleic acid sequences that encode the variant HBMYCNG,
gene product amino acid sequences, as depicted in FIG. 6;
(e) nucleic acid sequences that encode portions of the
10 HBMYCNG, gene product corresponding to functional domains
and individual exons; (f) nucleic acid sequences
comprising the novel complete gene sequence disclosed
herein, or portions thereof, that encode mutants of the
corresponding gene product in which all or a part of one
15 or more of the domains is deleted or altered; (g) nucleic
acid sequences that encode fusion proteins comprising the
HBMYCNG gene product, or one or more of its domains,
fused to a heterologous polypeptide; (h) nucleic acid
sequences within the HBMYCNG gene, as well as chromosome
20 sequences flanking that gene, that can be utilized as
part of the methods of the present invention for the
diagnosis or treatment of human disease; and (i) nucleic
acid sequences that hybridize to the above-described
sequences under stringent conditions. The nucleic acids
25 of the invention include, but are not limited to, cDNA
and genomic DNA sequences of the HBMYCNG gene.

The present invention also encompasses gene products
of the nucleic acid molecules listed above; i.e.,
proteins and/or polypeptides that are encoded by the
30 above-disclosed HBMYCNG nucleic acid molecules and are
expressed in recombinant host systems.

Antagonists and agonists of the HBMYCNG gene and/or
gene product disclosed herein are also included in the
present invention. Such antagonists and agonists will
35 include, for example, small molecules, large molecules,
and antibodies directed against the HBMYCNG gene product.
Antagonists and agonists of the invention also include

nucleotide sequences, such as antisense and ribozyme
 5 molecules, and gene or regulatory sequence replacement
 constructs, that can be used to inhibit or enhance
 expression of the disclosed HBMYCNG nucleic acid
 molecules.

The present invention further encompasses cloning
 10 vectors, including expression vectors, that contain the
 nucleic acid molecules of the invention and can be used
 to express those nucleic acid molecules in host
 organisms. The present invention also relates to host
 cells engineered to contain and/or express the nucleic
 15 acid molecules of the invention. Further, host organisms
 that have been transformed with these nucleic acid
 molecules are also encompassed in the present invention,
 e.g., transgenic animals, particularly transgenic
 non-human animals, and more particularly transgenic
 20 non-human mammals.

The present invention also relates to methods and
 compositions for the diagnosis of human disease involving
 cation, e.g., Ca^{2+} , sodium or potassium channel,
 dysfunction or lack of other ionic homeostasis including
 25 but not limited to, CNS disorders such as stroke, anxiety
 and depression, and degenerative neurological diseases,
 e.g., Alzheimer's disease or Parkinson's disease, or
 disorders such as cardiac disorders, e.g., arrhythmia,
 diabetes, chronic pain or other disorders such as
 30 hypercalcemia, hypercalciuria, or Ca^{2+} , sodium or
 potassium channel dysfunction that is associated with
 immunological disorders (GI) tract disorders, or renal or
 liver disease. The present invention further relates to
 methods and compositions useful for the diagnosis and
 35 treatment of diseases and conditions related to or
 involving the serotonin nervous system which participates
 in the control of anxiety, fear, depression, sleep and

5 pain. Accordingly, the present invention still further
relates to methods and compositions for the diagnosis of
anxiety and fear disorders, bipolar and major depression,
panic disorder, headaches, migraine, disorders of
circadian rhythmicity, stress, various sexual
dysfunctions including but not limited to erectile
10 dysfunction, neuroleptic-induced catalepsy, Rett syndrome
and aggressive behaviors.

Such methods comprise, for example, measuring
expression of the HBMYCNG gene in a patient sample, or
detecting a mutation in the gene in the genome of a
15 mammal, including a human, suspected of exhibiting ion
channel dysfunction. The nucleic acid molecules of the
invention can also be used as diagnostic hybridization
probes or as primers for diagnostic PCR analysis to
identify HBMYCNG gene mutations, allelic variations, or
20 regulatory defects, such as defects in the expression of
the gene. Such diagnostic PCR analyses can be used to
diagnose individuals with disorders associated with a
particular HBMYCNG gene mutation, allelic variation, or
regulatory defect. Such diagnostic PCR analyses can also
25 be used to identify individuals susceptible to ion
channel disorders.

Methods and compositions, including pharmaceutical
compositions, for the treatment of ion channel disorders
are also included in the invention. Such methods and
30 compositions are capable of modulating the level of
HBMYCNG gene expression and/or the level of activity of
the respective gene product. Such methods include, for
example, modulating the expression of the HBMYCNG gene
and/or the activity of the HBMYCNG gene product for the
35 treatment of a disorder that is mediated by a defect in
some other gene.

Such methods also include screening methods for the
5 identification of compounds that modulate the expression
of the nucleic acids and/or the activity of the
polypeptides of the invention, e.g., assays that measure
HBMYCNG mRNA and/or gene product levels, and assays that
measure levels of HBMYCNG activity, such as the ability
10 of the gene products to allow Ca^{2+} influx into cells.

For example, cellular and non-cellular assays are
known that can be used to identify compounds that
interact with the HBMYCNG gene and/or gene product, e.g.,
modulate the activity of the gene and/or bind to the gene
15 product. Such cell-based assays of the invention utilize
cells, cell lines, or engineered cells or cell lines that
express the gene product.

In one embodiment, such methods comprise contacting
a compound to a cell that expresses the HBMYCNG gene,
20 measuring the level of gene expression, gene product
expression, or gene product activity, and comparing this
level to the level of the HBMYCNG gene expression, gene
product expression, or gene product activity produced by
the cell in the absence of the compound, such that if the
25 level obtained in the presence of the compound differs
from that obtained in its absence, a compound that
modulates the expression of the HBMYCNG gene and/or the
synthesis or activity of the gene product has been
identified.

30 In an alternative embodiment, such methods comprise
administering a compound to a host organism, e.g., a
transgenic animal that expresses a HBMYCNG transgene or a
mutant HBMYCNG transgene, and measuring the level of
HBMYCNG gene expression, gene product expression, or gene
35 product activity. The measured level is compared to the
level of HBMYCNG gene expression, gene product
expression, or gene product activity in a host that is

not exposed to the compound, such that if the level
5 obtained when the host is exposed to the compound differs
from that obtained when the host is not exposed to the
compound, a compound that modulates the expression of the
HBMYCNG gene and/or the synthesis or activity of HBMYCNG
gene products has been identified.

10 The compounds identified by these methods include
therapeutic compounds that can be used as pharmaceutical
compositions to reduce or eliminate the symptoms of ion
channel disorders such as CNS disorders, e.g., stroke,
chronic pain, anxiety and depression, or degenerative
15 neurological diseases such as Alzheimer's disease or
Parkinson's disease, cardiac diseases or other
ion-related disorders such as hypercalcemia,
hypocalcemia, hypercalciuria, hypocalciuria, or ion
disorders that are associated with immunological
20 disorders, gastro-intestinal (GI) tract disorders, or
renal or liver disease. Compounds identified by these
methods further include compound useful for the treatment
of diseases and conditions related to or involving the
serotonin nervous system which participates in the
25 control of anxiety, fear, depression, sleep and pain.
Accordingly, compounds identified by these methods can be
used for the treatment of anxiety and fear disorders,
bipolar and major depression, panic disorder, headaches,
migraine, disorders of circadian rhythmicity, stress,
30 various sexual dysfunctions including but not limited to
erectile dysfunction, neuroleptic-induced catalepsy, Rett
syndrome and aggressive behaviors.

In another embodiment, screening methods are used
for the detection, isolation, and identification of
35 compounds which modulate the level of intracellular
cyclic nucleotides. In one example, cells expressing the
human HBMYCNG gene and a second biochemical activity

involved in cyclic nucleotide synthesis or degradation,
 5 including but not limited to a G-protein coupled
 receptor, are contacted with a test compound and the
 level of calcium, or other cation, influx is determined.
 Evaluation of calcium, or other cation, influx in the
 presence or absence of the test compound indicates
 10 whether that compound is an agonist or antagonist of
 cyclic nucleotide accumulation within the cell.

Similarly, in another embodiment, such an assay can
 be used to detect, isolate, and characterize the cognate
 ligand recognized by an "orphan" G-protein coupled
 15 receptor. In this embodiment, the cell expressing both
 the human HBMYCNG gene and the orphan G-protein coupled
 receptor is contacted with compounds and/or mixtures of
 compounds, and human HBMYCNG mediated calcium, or other
 cation, influx is determined with and without the test
 20 compounds. Presence of the cognate ligand for the
 "orphan" receptor is indicated by the intracellular
 synthesis of cAMP and/or cGMP mediated by the activated
 G-protein coupled receptor, leading to activation of the
 HBMYCNG cation channel and an increase in calcium, or
 25 other cation, influx into the cell.

4. DESCRIPTION OF THE FIGURES

FIG. 1. Nucleotide sequence (SEQ ID NO:1) and
 amino acid sequence (SEQ ID NO:2) of the full length cDNA
 30 for Human HBMYCNG. The ATG initiation codon for HBMYCNG
 translation is found at nucleotides 20-22, and the TAA
 termination codon is found at nucleotides 2012-2014.

FIG. 2. Conceptual translation of the open reading
 frame of the cDNA sequence of Figure 1, providing the
 35 amino acid sequence Human HBMYCNG (SEQ ID NO:2).

FIG. 3. Conceptual translation of nucleotide 20 to
 2011 of the 2186-nucleotide (SEQ ID NO:2), full length

Human HBMYCNG cDNA with the six transmembrane segments in
 5 bold and the ion pore underlined.

FIG. 4. Amino acid Sequence alignment of Human
 HBMYCNG (SEQ ID NO:2) and related rabbit (rACNG; gi
 433960), bovine (CNG2_BOS; gi 227199), murine
 (CNG2_mouse; gi 6671780), and rat (CNG2_RAT; gi 227120)
 10 cyclic nucleotide gated channels. Blackened areas
 represent identical amino acids and the gray highlighted
 residues indicate similar amino acids.

FIG. 5. Nucleotide sequence (SEQ ID NO:23) and
 amino acid sequence (SEQ ID NO:24) of a variant of the
 15 full length cDNA for Human HBMYCNG. The ATG initiation
 codon for the variant HBMYCNG translation is found at
 nucleotides 20-22, and the TAA termination codon is found
 at nucleotides 2012-2014.

FIG. 6. Conceptual translation of the open reading
 20 frame of the cDNA sequence of Figure 5, providing the
 amino acid sequence variant Human HBMYCNG (SEQ ID NO:24).

FIG. 7. Amino acid Sequence alignment of the Human
 HBMYCNG (SEQ ID NO:2) with the Human HBMYCNG variant (SEQ
 ID NO:24). Vertical bars ("|") represent identical amino
 25 acids. The threonine to isoleucine amino acid change in
 the Human HBMYCNG variant sequence at amino acid position
 442 of SEQ ID NO:24 is noted.

5. DETAILED DESCRIPTION OF THE INVENTION

30 The present invention relates to the isolation and
 identification of novel nucleic acid molecules and
 proteins and polypeptides for the formation or function
 of novel human ion channels. More specifically, the
 invention relates to a novel HBMYCNG human gene which
 35 encodes the corresponding HBMYCNG protein or biologically
 active derivatives or fragments thereof, involved in the
 formation or function of cation channels.

The HBMYCNG nucleic acid molecules of the present invention include isolated naturally-occurring or recombinantly-produced human HBMYCNG nucleic acid molecules, e.g., DNA molecules, cloned genes or degenerate variants thereof. The compositions of the invention also include isolated, naturally-occurring or recombinantly-produced human HBMYCNG protein or polypeptide.

Other embodiments of the invention include antibodies directed to the HBMYCNG protein or polypeptide of the invention and methods and compositions for the diagnosis and treatment of human diseases related to ion channel dysfunction as described below.

5.1. The HBMYCNG Nucleic Acids of the Invention

The complete HBMYCNG gene of the invention, HBMYCNG, is a novel, complete human nucleic acid molecule that encodes a protein or polypeptide involved in the formation or function of a novel human ion channel. Although this gene and the protein encoded therein display sequence and structural homology to other cation channel proteins known in the art, it is also known in the art that proteins displaying these homologies have significant differences in function, such as conductance and permeability, as well as differences in tissue expression, as well as co-expression, or not, of different CNG β -subunit polypeptides. As such, it is acknowledged in the art that nucleic acid molecules and the proteins encoded by those molecules sharing these homologies can still represent diverse, distinct and unique nucleic acids and proteins, respectively.

The HBMYCNG nucleic acid molecules of the invention include the following: (a) a nucleic acid molecule comprising the DNA sequence, HBMYCNG, as shown in FIG. 1

- or FIG. 5; (b) any nucleic acid sequence that encodes the
 5 amino acid sequence, HBMYCNG as shown in FIG. 2 or FIG.
 6; (c) any nucleic acid sequence that hybridizes to the
 complement of DNA sequences that encode the amino acid
 sequences of FIG. 2 or FIG.6 under highly stringent
 conditions, e.g., hybridization to filter-bound DNA in
 10 0.5 M NaHPO₄, 7% sodium dodecyl sulfate (SDS), 1 mM EDTA
 at 65°C, and washing in 0.1xSSC/0.1% SDS at 68°C (see,
 e.g., Ausubel F.M. et al., eds., 1989, Current Protocols
 in Molecular Biology, Vol. I, Green Publishing
 Associates, Inc., and John Wiley & sons, Inc., New York,
 15 at p. 2.10.3) or (d) any nucleic acid sequence that
 hybridizes to the complement of DNA sequences that encode
 the amino acid sequences of FIG. 2 or FIG.6, under less
 stringent conditions, such as moderately stringent
 conditions, e.g., washing in 0.2xSSC/0.1% SDS at 42°C
 20 (Ausubel et al., 1989, *supra*), and which encodes a gene
 product functionally equivalent to a HBMYCNG gene product
 encoded by the deposited sequences or the sequence
 depicted in FIG. 2 or FIG. 6. "Functionally equivalent"
 as used herein refers to any protein capable of
 25 exhibiting a substantially similar *in vivo* or *in vitro*
 activity as the HBMYCNG gene product encoded by the
 HBMYCNG nucleic acid molecules described herein, e.g.,
 ion channel formation or function. For the purposes of
 the present invention, the HBMYCNG nucleic acid as
 30 depicted in FIG. 1 is functionally equivalent to the
 HBMYCNG nucleic acid as depicted in FIG. 5.

As used herein, the term "HBMYCNG nucleic acid
 molecule" may also refer to fragments and/or degenerate
 variants of DNA sequences (a) through (d), including
 35 naturally occurring variants or mutant alleles thereof.
 Such fragments include, for example, nucleotide sequences
 that encode portions of the HBMYCNG protein that

correspond to functional domains of the protein. One
5 embodiment of such a HBMYCNG nucleic acid fragment
comprises a nucleic acid that encodes the fifth and sixth
transmembrane segments of the HBMYCNG protein, including
the predicted pore loop (see FIG. 3).

Additionally, the HBMYCNG nucleic acid molecules of
10 the invention include isolated nucleic acid molecules,
preferably DNA molecules, that hybridize under highly
stringent or moderately stringent hybridization
conditions to at least about 6, preferably at least about
12, and more preferably at least about 18, consecutive
15 nucleotides of the nucleic acid sequences of (a) through
(d), identified *supra*.

The HBMYCNG nucleic acid molecules of the invention
also include nucleic acid molecules, preferably DNA
molecules, that hybridize to, and are therefore
20 complements of, the DNA sequences of (a) through (d),
supra. Such hybridization conditions may be highly
stringent or moderately stringent, as described above. In
those instances in which the nucleic acid molecules are
deoxyoligonucleotides ("oligos"), highly stringent
25 conditions may include, e.g., washing in 6xSSC/0.05%
sodium pyrophosphate at 37°C (for 14-base oligos), 48°C
(for 17-base oligos), 55°C (for 20-base oligos), and 60°C
(for 23-base oligos). These nucleic acid molecules may
encode or act as HBMYCNG antisense molecules useful, for
30 example, in HBMYCNG gene regulation or as antisense
primers in amplification reactions of HBMYCNG nucleic
acid sequences. Further, such sequences may be used as
part of ribozyme and/or triple helix sequences, also
useful for HBMYCNG gene regulation. Still further, such
35 molecules may be used as components of diagnostic methods
whereby, for example, the presence of a particular
HBMYCNG allele or alternatively spliced HBMYCNG

transcript responsible for causing or predisposing one to
 5 a disorder involving ion channel dysfunction may be
 detected.

Typically, the HBMYCNG nucleic acids of the
 invention should exhibit at least about 90% overall
 homology at the nucleotide level, and more preferably at
 10 least about 95% overall homology to the nucleic acid
 sequence of FIG. 1.

Also included within the HBMYCNG nucleic acids of
 the invention are nucleic acid molecules, preferably DNA
 molecules, comprising an HBMYCNG nucleic acid, as
 15 described herein, operatively linked to a nucleotide
 sequence encoding a heterologous protein or peptide.

To determine the percent identity of two nucleic
 acid sequences or of two amino acid sequences, the
 sequences are aligned for optimal comparison purposes
 20 (e.g., gaps can be introduced in the sequence of a first
 amino acid or nucleic acid sequence for optimal alignment
 with a second amino acid or nucleic acid sequence). The
 amino acid residues or nucleotides at corresponding amino
 acid positions or nucleotide positions are then compared.
 25 When a position in the first sequence is occupied by the
 same amino acid residue or nucleotide as the
 corresponding position in the second sequence, then the
 molecules are identical at that position. The percent
 identity between the two sequences is a function of the
 30 number of identical positions shared by the sequences
 (i.e., % identity = number of identical overlapping
 positions/total number of positions x 100%). In one
 embodiment, the two sequences are the same length.

The determination of percent identity between two
 35 sequences can also be accomplished using a mathematical
 algorithm. A preferred, non-limiting example of a
 mathematical algorithm utilized for the comparison of two

sequences is the algorithm of Karlin and Altschul, 1990,
 5 Proc. Natl. Acad. Sci. U.S.A. 87:2264-2268, modified as
 in Karlin and Altschul, 1993, Proc. Natl. Acad. Sci.
 U.S.A. 90: 5873-5877. Such an algorithm is incorporated
 into the NBLAST and XBLAST programs of Altschul et al.,
 1990, J. Mol. Biol. 215: 403. BLAST nucleic acid searches
 10 can be performed with the NBLAST nucleic acid program
 parameters set, e.g., for score=100, wordlength=12 to
 obtain nucleic acid sequences homologous to a nucleic
 acid molecule of the present invention. BLAST polypeptide
 searches can be performed with the XBLAST program
 15 parameters set, e.g., to score=50, wordlength=3 to obtain
 amino acid sequences homologous to a polypeptide molecule
 of the present invention. To obtain gapped alignments for
 comparison purposes, Gapped BLAST can be utilized as
 described in Altschul et al., 1997, Nucleic Acids Res.
 20 25: 3389-3402. Alternatively, PSI-BLAST can be used to
 perform an iterated search which detects distant
 relationships between molecules (Id.). When utilizing
 BLAST, Gapped BLAST, and PSI-Blast programs, the default
 parameters of the respective programs (e.g., of XBLAST
 25 and NBLAST) can be used (e.g.,
<http://www.ncbi.nlm.nih.gov>). Another preferred,
 non-limiting example of a mathematical algorithm utilized
 for the comparison of sequences is the algorithm of Myers
 and Miller, 1988, CABIOS 4:11-17. Such an algorithm is
 30 incorporated in the ALIGN program (version 2.0) which is
 part of the GCG sequence alignment software package. When
 utilizing the ALIGN program for comparing amino acid
 sequences, a PAM120 weight residue table, a gap length
 penalty of 12, and a gap penalty of 4 can be used. Still
 35 another preferred algorithm for the comparison of
 polypeptide sequences is that of Thompson et al.,

designated CLUSTALW, which is disclosed in Thompson et al. 1994 Nucleic Acids Research 2(22): 4673-80.

The percent identity between two sequences can be determined using techniques similar to those described above, with or without allowing gaps. In calculating percent identity, typically only exact matches are counted.

Moreover, due to the degeneracy of the genetic code, other DNA sequences that encode substantially the amino acid sequence of HBMYCNG may be used in the practice of the present invention for the cloning and expression of HBMYCNG polypeptides. Such DNA sequences include those that are capable of hybridizing to the HBMYCNG nucleic acids of this invention under stringent (high or moderate) conditions, or that would be capable of hybridizing under stringent conditions but for the degeneracy of the genetic code.

Altered HBMYCNG DNA sequences that may be used in accordance with the invention include deletions, additions or substitutions of different nucleotide residues resulting in a nucleic acid molecule that encodes the same or a functionally equivalent gene product as those described *supra*. The gene product itself may contain deletions, additions or substitutions of amino acid residues within the HBMYCNG protein sequence, which result in a silent change, thus producing a functionally equivalent HBMYCNG polypeptide. Such amino acid substitutions may be made on the basis of similarity in polarity, charge, solubility, hydrophobicity, hydrophilicity, and/or the amphipatic nature of the residues involved. For example, negatively-charged amino acids include aspartic acid and glutamic acid; positively-charged amino acids include lysine and arginine; amino acids with uncharged polar head groups

having similar hydrophilicity values include the
5 following: leucine, isoleucine, valine; glycine, aniline;
asparagine, glutamine; serine, threonine; phenylalanine,
tyrosine. A functionally equivalent HBMYCNG polypeptide
can include a polypeptide which displays the same type of
biological activity (e.g., cation channel) as the native
10 HBMYCNG protein, but not necessarily to the same extent.

The nucleic acid molecules or sequences of the
invention may be engineered in order to alter the HBMYCNG
coding sequence for a variety of ends including but not
limited to alterations that modify processing and
15 expression of the gene product. For example, mutations
may be introduced using techniques which are well known
in the art, e.g., site-directed mutagenesis, to insert
new restriction sites, to alter glycosylation patterns,
phosphorylation, etc. For example, in certain expression
20 systems such as yeast, host cells may over-glycosylate
the gene product. When using such expression systems, it
may be preferable to alter the HBMYCNG coding sequence to
eliminate any N-linked glycosylation sites.

In another embodiment of the invention, the HBMYCNG
25 nucleic acid or a modified HBMYCNG sequence may be
ligated to a heterologous sequence to encode a fusion
protein. The fusion protein may be engineered to contain
a cleavage site located between the HBMYCNG sequence and
the heterologous protein sequence, so that the HBMYCNG
30 protein can be cleaved away from the heterologous moiety.

The HBMYCNG nucleic acid molecules of the invention
can also be used as hybridization probes for obtaining
HBMYCNG cDNAs or genomic HBMYCNG DNA. In addition, the
nucleic acids of the invention can be used as primers in
35 PCR amplification methods to isolate HBMYCNG cDNAs and
genomic DNA, e.g., from other species.

The HBMYCNG gene sequences of the invention may also
5 used to isolate mutant HBMYCNG gene alleles. Such mutant
alleles may be isolated from individuals either known or
proposed to have a genotype related to ion channel
dysfunction. Mutant alleles and mutant allele gene
products may then be utilized in the screening,
10 therapeutic and diagnostic systems described in Section
5.4., *infra*. Additionally, such HBMYCNG gene sequences
can be used to detect HBMYCNG gene regulatory (e.g.,
promoter) defects which can affect ion channel function.

A cDNA of a mutant HBMYCNG gene may be isolated, for
15 example, by using PCR, a technique which is well known to
those of skill in the art (see, e.g., U.S. Patent No.
4,683,202). The first cDNA strand may be synthesized by
hybridizing an oligo-dT oligonucleotide to mRNA isolated
from tissue known or suspected to be expressed in an
20 individual putatively carrying the mutant HBMYCNG allele,
and by extending the new strand with reverse
transcriptase. The second strand of the cDNA is then
synthesized using an oligonucleotide that hybridizes
specifically to the 5' end of the normal gene. Using
25 these two primers, the product is then amplified via PCR,
cloned into a suitable vector, and subjected to DNA
sequence analysis through methods well known in the art.
By comparing the DNA sequence of the mutant HBMYCNG
allele to that of the normal HBMYCNG allele, the
30 mutation(s) responsible for the loss or alteration of
function of the mutant HBMYCNG gene product can be
ascertained.

Alternatively, a genomic library can be constructed
using DNA obtained from an individual suspected of or
35 known to carry the mutant HBMYCNG allele, or a cDNA
library can be constructed using RNA from a tissue known,
or suspected, to express the mutant HBMYCNG allele. The

normal HBMYCNG gene or any suitable fragment thereof may
5 then be labeled and used as a probe to identify the
corresponding mutant HBMYCNG allele in such libraries.
Clones containing the mutant HBMYCNG gene sequences may
then be purified and subjected to sequence analysis
according to methods well known in the art.

10 According to another embodiment, an expression
library can be constructed utilizing cDNA synthesized
from, for example, RNA isolated from a tissue known, or
suspected, to express a mutant HBMYCNG allele in an
individual suspected of or known to carry such a mutant
15 allele. Gene products made by the putatively mutant
tissue may be expressed and screened using standard
antibody screening techniques in conjunction with
antibodies raised against the normal HBMYCNG gene
product, as described in Section 5.3, *supra*. For
20 screening techniques, see, for example, Harlow, E. and
Lane, eds., 1988, "Anti-bodies: A Laboratory Manual",
Cold Spring Harbor Press, Cold Spring Harbor.

In cases where a HBMYCNG mutation results in an
expressed gene product with altered function (e.g., as a
25 result of a missense or a frameshift mutation), a
polyclonal set of anti-HBMYCNG gene product antibodies
are likely to cross-react with the mutant HBMYCNG gene
product. Library clones detected via their reaction with
such labeled antibodies can be purified and subjected to
30 sequence analysis according to methods well known to
those of skill in the art.

In an alternate embodiment of the invention, the
coding sequence of HBMYCNG can be synthesized in whole or
in part, using chemical methods well known in the art,
35 based on the nucleic acid and/or amino acid sequences of
the HBMYCNG genes and proteins disclosed herein. See, for
example, Caruthers et al., 1980, Nuc. Acids Res. Symp.

- Ser. 7: 215-233; Crea and Horn, 1980, Nuc. Acids Res.
 5 9(10): 2331; Matteucci and Caruthers, 1980, Tetrahedron
 Letters 21: 719; and Chow and Kempe, 1981, Nuc. Acids
 Res. 9(12): 2807-2817.

The invention also encompasses (a) DNA vectors that
 contain any of the foregoing HBMYCNG sequences and/or
 10 their complements; (b) DNA expression vectors that
 contain any of the foregoing HBMYCNG coding sequences
 operatively associated with a regulatory element that
 directs the expression of the coding sequences; and (c)
 genetically engineered host cells that contain any of the
 15 foregoing HBMYCNG coding sequences operatively associated
 with a regulatory element that directs the expression of
 the coding sequences in the host cell. As used herein,
 regulatory elements include, but are not limited to
 inducible and non-inducible promoters, enhancers,
 20 operators and other elements known to those skilled in
 the art that drive and regulate expression. Such
 regulatory elements include but are not limited to the
 cytomegalovirus hCMV immediate early gene, the early or
 late promoters of SV40 adenovirus, the *lac* system, the
 25 *trp* system, the *TAC* system, the *TRC* system, the major
 operator and promoter regions of phage A, the control
 regions of fd coat protein, the promoter for
 3-phosphoglycerate kinase, the promoters of acid
 phosphatase, and the promoters of the yeast α -mating
 30 factors.

The invention still further includes nucleic acid
 analogs, including but not limited to peptide nucleic
 acid analogues, equivalent to the nucleic acid molecules
 described herein. "Equivalent" as used in this context
 35 refers to nucleic acid analogs that have the same primary
 base sequence as the nucleic acid molecules described
 above. Nucleic acid analogs and methods for the synthesis

of nucleic acid analogs are well known to those of skill
 5 in the art. See, e.g., Egholm, M. et al., 1993, Nature
 365:566-568; and Perry-O'Keefe, H. et al., 1996, Proc.
 Natl. Acad. USA 93:14670-14675.

5.2. HBMYCNG Proteins and Polypeptides of the Invention

10 The HBMYCNG nucleic acid molecules of the invention
 may be used to generate recombinant DNA molecules that
 direct the expression in appropriate host cells of
 HBMYCNG polypeptides, including the full-length HBMYCNG
 protein, functionally active or equivalent HBMYCNG
 15 proteins and polypeptides, e.g., mutated, truncated or
 deleted forms of HBMYCNG, peptide fragments of HBMYCNG,
 or HBMYCNG fusion proteins. A functionally equivalent
 HBMYCNG polypeptide can include a polypeptide which
 displays the same type of biological activity (e.g.,
 20 cation channel formation and/or function) as the native
 HBMYCNG protein, but not necessarily to the same extent.

In a preferred embodiment, the proteins and
 polypeptides of the invention include the HBMYCNG amino
 acid sequence depicted in FIG. 2, which corresponds to
 25 the conceptual translation of the nucleotide sequence
 spanning residues 20 to 2011 of the cDNA sequence of
 HBMYCNG, as depicted in FIG. 1. This amino acid sequence
 includes six transmembrane domains and an overall
 topology that is conserved in CNG ion channels.

30 In other embodiments of the present invention the
 proteins and polypeptides of the invention include the
 HBMYCNG amino acid sequence depicted in FIG. 2 except for
 the initial methionine residue; i.e., a polypeptide
 having an amino acid sequence corresponding to amino
 35 acids 2 through 664 the amino acid sequence of FIG. 2,
 which corresponds to the conceptual translation of the

nucleotide sequence spanning residues 23 to 2011 of the
 5 cDNA sequence of HBMYCNG, as depicted in FIG. 1.

The HBMYCNG amino acid sequence of FIG. 2, which has
 a calculated molecular weight of 75.9 kDa, is homologous
 to four cyclic nucleotide gated proteins. A comparison of
 the HBMYCNG amino acid sequence with that of rabbit
 10 (rACNG; gi 433960), bovine (CNG2_BOS; gi 227199), mouse
 (CNG2_mouse; gi 6671780), and rat (CNG2_RAT; gi 227120)
 cyclic nucleotide gated channels from rabbit is presented
 in FIG. 4. The amino acid sequences for Human HBMYCNG and
 for rabbit aorta rCNG displayed 95.633% similarity and
 15 93.675% identity; the amino acid sequences for Human
 HBMYCNG and for bovine olfactory CNG2_BOVIN displayed
 95.324% similarity and 93.213% identity; the amino acid
 sequences for Human HBMYCNG and for murine olfactory
 CNG2_MOUSE displayed 94.260% similarity and 93.051%
 20 identity; and the amino acid sequences for Human HBMYCNG
 and for rat olfactory CNG2_RAT displayed 94.109%
 similarity and 92.598% identity.

The HBMYCNG proteins and polypeptides of the
 invention include peptide fragments of HBMYCNG, e.g.,
 25 peptides corresponding to one or more domains of the
 protein, mutated, truncated or deleted forms of the
 proteins and polypeptides, as well as HBMYCNG fusion
 proteins, all of which derivatives of HBMYCNG can be
 obtained by techniques well known in the art, given the
 30 HBMYCNG nucleic acid and amino acid sequences disclosed
 herein.

As noted in Section 5.1, *supra*, the proteins and
 polypeptides of the invention may contain deletions,
 additions or substitutions of amino acid residues within
 35 the HBMYCNG protein sequence, which result in a silent
 change, thus producing a functionally equivalent HBMYCNG
 polypeptide. Such amino acid substitutions may be made on

the basis of similarity in polarity, charge, solubility,
5 hydrophobicity, hydrophilicity, and/or the amphipatic
nature of the residues involved. For example,
negatively-charged amino acids include aspartic acid and
glutamic acid; positively-charged amino acids include
lysine, arginine and histidine; amino acids with
10 uncharged polar head groups having similar hydrophilicity
values include the following: leucine, isoleucine,
valine, glycine, alanine, asparagine, glutamine, serine,
threonine, phenylalanine, tyrosine.

Mutated or altered forms of the HBMYCNG proteins and
15 polypeptides of the invention can be obtained using
either random mutagenesis techniques or site-directed
mutagenesis techniques well known in the art or by
chemical methods, e.g., protein synthesis techniques (see
Section 5.1, *supra*). Mutant HBMYCNG proteins or
20 polypeptides can be engineered so that regions important
for function are maintained, while variable residues are
altered, e.g., by deletion or insertion of an amino acid
residue(s) or by the substitution of one or more
different amino acid residues. For example, conservative
25 alterations at the variable positions of a polypeptide
can be engineered to produce a mutant HBMYCNG polypeptide
that retains the function of HBMYCNG. Non-conservative
alterations of variable regions can be engineered to
alter HBMYCNG function, if desired. Alternatively, in
30 those cases where modification of function (either to
increase or decrease function) is desired, deletion or
non-conservative alterations of conserved regions of the
polypeptide may be engineered.

Fusion proteins containing HBMYCNG amino acid
35 sequences can also be obtained by techniques known in the
art, including genetic engineering and chemical protein
synthesis techniques. According to a preferred

embodiment, the fusion proteins of the invention are
5 encoded by an isolated nucleic acid molecule comprising
an HBMYCNG nucleic acid of the invention that encodes a
polypeptide with an activity of a HBMYCNG protein, or a
fragment thereof, linked in frame and uninterrupted by
stop codons to a nucleotide sequence that encodes a
10 heterologous protein or peptide.

The fusion proteins of the invention include those
that contain the full length HBMYCNG amino acid sequence,
an HBMYCNG peptide sequence, e.g., encoding one or more
functional domains, a mutant HBMYCNG amino acid sequence
15 or a truncated HBMYCNG amino acid sequence linked to an
unrelated protein or polypeptide sequence. Such fusion
proteins include but are not limited to IgFc fusions
which stabilize the HBMYCNG fusion protein and may
prolong half-life of the protein *in vivo* or fusions to an
20 enzyme, fluorescent protein or luminescent protein that
provides a marker function.

According to a preferred embodiment, the HBMYCNG
nucleic acid molecules of the invention may be used to
generate recombinant DNA molecules that direct the
25 expression of HBMYCNG polypeptides, including the
full-length HBMYCNG protein, e.g., HBMYCNG or
functionally active or equivalent HBMYCNG peptides
thereof, or HBMYCNG fusion proteins in appropriate host
cells.

30 In order to express a biologically active HBMYCNG
polypeptide, a nucleic acid molecule coding for the
polypeptide, or a functional equivalent thereof as
described in Section 5.1, *supra*, is inserted into an
appropriate expression vector, i.e., a vector which
35 contains the necessary elements for the transcription and
translation of the inserted coding sequence. The HBMYCNG
gene products so produced, as well as host cells or cell

lines transfected or transformed with recombinant HBMYCNG
 5 expression vectors, can be used for a variety of
 purposes. These include but are not limited to generating
 antibodies (i.e., monoclonal or polyclonal) that bind to
 the HBMYCNG protein, including those that competitively
 inhibit binding and thus can "neutralize" HBMYCNG
 10 activity, and the screening and selection of HBMYCNG
 analogs or ligands.

Methods which are well known to those skilled in the
 art are used to construct expression vectors containing
 the HBMYCNG coding sequences of the invention and
 15 appropriate transcriptional and translational control
 signals. These methods include *in vitro* recombinant DNA
 techniques, synthetic techniques and *in vivo*
 recombination/genetic recombination. See, for example,
 the techniques described in Maniatis et al., 1989,
 20 Molecular Cloning, A Laboratory Manual, Cold Spring
 Harbor Laboratory, N.Y. and Ausubel et al., 1989, Current
 Protocols in Molecular Biology, Greene Publishing
 Associates and Wiley Interscience, N.Y. See also Sambrook
 et al., 1989, Molecular Cloning, A Laboratory Manual,
 25 Cold Spring Harbor Press, N.Y.

A variety of host-expression vector systems may be
 used to express the HBMYCNG coding sequences of this
 invention. Such host-expression systems represent
 vehicles by which the coding sequences of interest may be
 30 produced and subsequently purified, but also represent
 cells which may, when transformed or transfected with the
 appropriate nucleotide coding sequences, exhibit the
 corresponding HBMYCNG gene products *in situ* and/or
 function *in vivo*. These hosts include but are not limited
 35 to microorganisms such as bacteria (e.g., *E. coli*, *B.*
subtilis) transformed with recombinant bacteriophage DNA,
 plasmid DNA or cosmid DNA expression vectors containing

the HBMYCNG coding sequences; yeast (e.g., *Saccharomyces*,
 5 *Pichia*) transformed with recombinant yeast expression
 vectors containing the HBMYCNG coding sequence; insect
 cell systems infected with recombinant virus expression
 vectors (e.g., *baculovirus*) containing the HBMYCNG coding
 sequence; plant cell systems infected with recombinant
 10 virus expression vectors (e.g., cauliflower mosaic virus,
 CaMV; tobacco mosaic virus, TMV) or transformed with
 recombinant plasmid expression vectors (e.g., Ti plasmid)
 containing the HBMYCNG coding sequence; or mammalian cell
 systems (e.g., COS, CHO, BHK, 293, 3T3) harboring
 15 recombinant expression constructs containing promoters
 derived from the genome of mammalian cells (e.g., the
 metallothionein promoter) or from mammalian viruses
 (e.g., the adenovirus late promoter or vaccinia virus
 7.5K promoter).

20 The expression elements of these systems can vary in
 their strength and specificities. Depending on the
 host/vector system utilized, any of a number of suitable
 transcription and translation elements, including
 constitutive and inducible promoters, may be used in the
 25 expression vector. For example, when cloning in bacterial
 systems, inducible promoters such as pL of bacteriophage
 ?, plac, ptrp, ptac (ptrp-lac hybrid promoter) and the
 like may be used; when cloning in insect cell systems,
 promoters such as the baculovirus polyhedrin promoter may
 30 be used; when cloning in plant cell systems, promoters
 derived from the genome of plant cells (e.g., heat shock
 promoters; the promoter for the small subunit of RUBISCO;
 the promoter for the chlorophyll a/b binding protein) or
 from plant viruses (e.g., the 35S RNA promoter of CaMV;
 35 the coat protein promoter of TMV) may be used; when
 cloning in mammalian cell systems, promoters derived from
 the genome of mammalian cells (e.g., metallothionein

promoter) or from mammalian viruses (e.g., the adenovirus
5 late promoter; the vaccinia virus 7.5K promoter) may be
used; when generating cell lines that contain multiple
copies of the HBMYCNG DNA, SV40-, BPV- and EBV-based
vectors may be used with an appropriate selectable
marker.

10 In bacterial systems, a number of expression vectors
may be advantageously selected depending upon the use
intended for the HBMYCNG expressed. For example, when
large quantities of an HBMYCNG polypeptide are to be
produced, e.g., for the generation of antibodies or the
15 production of the HBMYCNG gene product, vectors which
direct the expression of high levels of fusion protein
products that are readily purified may be desirable. Such
vectors include but are not limited to the *E. coli*
expression vector pUR278 (Ruther et al., 1983, EMBO J. 2:
20 1791), in which the HBMYCNG coding sequence may be
ligated into the vector in frame with the lacZ coding
region so that a hybrid HBMYCNG/lacZ protein is produced;
pIN vectors (Inouye & Inouye, 1985, Nucleic Acids Res.
13: 3101-3109; Van Heeke & Schuster, 1989, J. Biol. Chem.
25 264: 5503-5509); and the like. pGEX vectors may also be
used to express foreign polypeptides as fusion proteins
with glutathione S-transferase (GST). In general, such
fusion proteins are soluble and can easily be purified
from lysed cells by affinity chromatography, e.g.,
30 adsorption to glutathione-agarose beads followed by
elution in the presence of free glutathione. The pGEX
vectors are designed to include thrombin or factor Xa
protease cleavage sites so that the cloned polypeptide of
interest can be released from the GST moiety. See also
35 Booth et al., 1988, Immunol. Lett. 19: 65-70; and
Gardella et al., 1990, J. Biol. Chem. 265: 15854-15859;
Pritchett et al., 1989, Biotechniques 7: 580.

In yeast, a number of vectors containing
 5 constitutive or inducible promoters may be used. For a
 review, see Current Protocols in Molecular Biology, Vol.
 2, 1988, Ed. Ausubel et al., Greene Publish. Assoc. &
 Wiley Interscience, Ch. 13; Grant et al., 1987,
 Expression and Secretion Vectors for Yeast, in Methods in
 10 Enzymology, Eds. Wu & Grossman, 1987, Acad. Press, N.Y.,
 Vol. 153, pp. 516-544; Glover, 1986, DNA Cloning, Vol.
 II, IRL Press, Wash., D.C., Ch. 3; and Bitter, 1987,
 Heterologous Gene Expression in Yeast, Methods in
 Enzymology, Eds. Berger & Kimmel, Acad. Press, N.Y., Vol.
 15 152, pp. 673-684; and The Molecular Biology of the Yeast
 Saccharomyces, 1982, Cold Spring Harbor Press, Vols. I
 and II.

In an insect system, *Autographa californica* nuclear
 polyhydrosis virus (AcNPV) can be used as a vector to
 20 express foreign genes. The virus grows in *Spodoptera*
frugiperda cells. The HBMYCNG coding sequence may be
 cloned into non-essential regions (for example, the
 polyhedrin gene) of the virus and placed under control of
 an AcNPV promoter (for example, the polyhedrin promoter).
 25 Successful insertion of the HBMYCNG coding sequence will
 result in inactivation of the polyhedrin gene and
 production of non-occluded recombinant virus (i.e., virus
 lacking the proteinaceous coat coded for by the
 polyhedrin gene). These recombinant viruses can then be
 30 used to infect *Spodoptera frugiperda* cells in which the
 inserted gene is expressed (see e.g., Smith et al., 1983,
 J. Virol. 46: 584; Smith, U.S. Patent No. 4,215,051).

In mammalian host cells, a number of viral-based
 expression systems may be utilized. In cases where an
 35 adenovirus is used as an expression vector, the HBMYCNG
 coding sequence may be ligated to an adenovirus
 transcription/translation control complex, e.g., the late

promoter and tripartite leader sequence. This chimeric
 5 gene may then be inserted in the adenovirus genome by *in vitro* or *in vivo* recombination. Insertion in a non-essential region of the viral genome (e.g., region E1 or E3) will result in a recombinant virus that is viable and capable of expressing HBMYCNG in infected hosts (see,
 10 e.g., Logan & Shenk, 1984, Proc. Natl. Acad. Sci. (USA) 81: 3655-3659). Alternatively, the vaccinia 7.5K promoter may be used (see, e.g., Mackett et al., 1982, Proc. Natl. Acad. Sci. (USA) 79: 7415-7419; Mackett et al., 1984, J. Virol. 49: 857-864; Panicali et al., 1982, Proc. Natl.
 15 Acad. Sci. 79: 4927-4931).

Specific initiation signals may also be required for efficient translation of inserted HBMYCNG coding sequences. These signals include the ATG initiation codon and adjacent sequences. In cases where the entire HBMYCNG
 20 gene, including its own initiation codon and adjacent sequences, is inserted into the appropriate expression vector, no additional translational control signals may be needed. However, in cases where only a portion of the HBMYCNG coding sequence is inserted, exogenous
 25 translational control signals, including the ATG initiation codon, must be provided. Furthermore, the initiation codon must be in phase with the reading frame of the HBMYCNG coding sequence to ensure translation of the entire insert. These exogenous translational control
 30 signals and initiation codons can be of a variety of origins, both natural and synthetic. The efficiency of expression may be enhanced by the inclusion of appropriate transcription enhancer elements, transcription terminators, etc. (see, e.g., Bittner et
 35 al., 1987, Methods in Enzymol. 153:516-544).

In addition, a host cell strain may be chosen which modulates the expression of the inserted sequences, or

modifies and processes the gene product in the specific
5 fashion desired. Such modifications (e.g., glycosylation)
and processing (e.g., cleavage) of protein products may
be important for the function of the protein. Different
host cells have characteristic and specific mechanisms
for the post-translational processing and modification of
10 proteins. Appropriate cells lines or host systems can be
chosen to ensure the correct modification and processing
of the foreign protein expressed. To this end, eukaryotic
host cells which possess the cellular machinery for
proper processing of the primary transcript,
15 glycosylation, and phosphorylation of the gene product
may be used. Such mammalian host cells include but are
not limited to CHO, VERO, BHK, HeLa, COS, MDCK, 293,
WI38, etc.

For long-term, high-yield production of recombinant
20 proteins, stable expression is preferred. For example,
cell lines which stably express the HBMYCNG polypeptides
of this invention may be engineered. Thus, rather than
using expression vectors which contain viral origins of
replication, host cells can be transformed with HBMYCNG
25 nucleic acid molecules, e.g., DNA, controlled by
appropriate expression control elements (e.g., promoter,
enhancer, sequences, transcription terminators,
polyadenylation sites, etc.), and a selectable marker.
Following the introduction of foreign DNA, engineered
30 cells may be allowed to grow for 1-2 days in an enriched
media, and then are switched to a selective media. The
selectable marker in the recombinant plasmid confers
resistance to the selection and allows cells to stably
integrate the plasmid into their chromosomes and grow to
35 form foci which in turn can be cloned and expanded into
cell lines. This method may advantageously be used to
engineer cell lines which express HBMYCNG polypeptides on

the cell surface. Such engineered cell lines are particularly useful in screening for HBMYCNG analogs or ligands.

In instances where the mammalian cell is a human cell, among the expression systems by which the HBMYCNG nucleic acid sequences of the invention can be expressed are human artificial chromosome (HAC) systems (see, e.g., Harrington et al., 1997, *Nature Genetics* 15: 345-355).

HBMYCNG gene products can also be expressed in transgenic animals such as mice, rats, rabbits, guinea pigs, pigs, micro-pigs, sheep, goats, and non-human primates, e.g., baboons, monkeys, and chimpanzees. The term "transgenic" as used herein refers to animals expressing HBMYCNG nucleic acid sequences from a different species (e.g., mice expressing human HBMYCNG nucleic acid sequences), as well as animals that have been genetically engineered to overexpress endogenous (i.e., same species) HBMYCNG nucleic acid sequences or animals that have been genetically engineered to no longer express endogenous HBMYCNG nucleic acid sequences (i.e., "knock-out" animals), and their progeny.

Transgenic animals according to this invention may be produced using techniques well known in the art, including but not limited to pronuclear microinjection (Hoppe, P.C. and Wagner, T.E., 1989, U.S. Pat. No. 4,873,191); retrovirus mediated gene transfer into germ lines (Van der Putten et al., 1985, *Proc. Natl. Acad. Sci., USA* 82: 6148-6152); gene targeting in embryonic stem cells (Thompson et al., 1989, *Cell* 56: 313-321); electroporation of embryos (Lo, 1983, *Mol Cell. Biol.* 3: 1803-1814); and sperm-mediated gene transfer (Lavitrano et al., 1989, *Cell* 57: 717-723); etc. For a review of such techniques, see Gordon, 1989, *Transgenic Animals*, *Intl. Rev. Cytol.* 115: 171-229.

In addition, any technique known in the art may be
5 used to produce transgenic animal clones containing a
HBMYCNG transgene, for example, nuclear transfer into
enucleated oocytes of nuclei from cultured embryonic,
fetal or adult cells induced to quiescence (Campbell et
al., 1996, Nature 380: 64-66; Wilmut et al., 1997, Nature
10 385: 810-813).

Host cells which contain the HBMYCNG coding sequence
and which express a biologically active gene product may
be identified by at least four general approaches; (a)
DNA-DNA or DNA-RNA hybridization; (b) the presence or
15 absence of "marker" gene functions; (c) assessing the
level of transcription as measured by the expression of
HBMYCNG mRNA transcripts in the host cell; and (d)
detection of the gene product as measured by immunoassay
or by its biological activity.

20 In the first approach, the presence of the HBMYCNG
coding sequence inserted in the expression vector can be
detected by DNA-DNA or DNA-RNA hybridization using probes
comprising nucleotide sequences that are homologous to
the HBMYCNG coding sequence, respectively, or portions or
25 derivatives thereof.

In the second approach, the recombinant expression
vector/host system can be identified and selected based
upon the presence or absence of certain "marker" gene
functions. For example, if the HBMYCNG coding sequence is
30 inserted within a marker gene sequence of the vector,
recombinants containing the HBMYCNG coding sequence can
be identified by the absence of the marker gene function.
Alternatively, a marker gene can be placed in tandem with
the HBMYCNG sequence under the control of the same or
35 different promoter used to control the expression of the
HBMYCNG coding sequence. Expression of the marker in

response to induction or selection indicates expression
 5 of the HBMYCNG coding sequence.

Selectable markers include resistance to
 antibiotics, resistance to methotrexate, transformation
 phenotype, and occlusion body formation in baculovirus.
 In addition, thymidine kinase activity (Wigler et al.,
 10 1977, Cell 11: 223) hypoxanthine-guanine
 phosphoribosyltransferase (Szybalska & Szybalski, 1962,
 Proc. Natl. Acad. Sci. USA 48: 2026), and adenine
 phosphoribosyltransferase (Lowy et al., 1980, Cell 22:
 817) genes can be employed in tk-, hgprt- or aprt- cells,
 15 respectively. Also, antimetabolite resistance can be used
 as the basis of selection for dhfr, which confers
 resistance to methotrexate (Wigler et al., 1980, Proc.
 Natl. Acad. Sci. USA 77: 3567; O'Hare et al., 1981, Proc.
 Natl. Acad. Sci. USA 78: 1527); gpt, which confers
 20 resistance to mycophenolic acid (Mulligan & Berg, 1981,
 Proc. Natl. Acad. Sci. USA 78: 2072); neo, which confers
 resistance to the aminoglycoside G-418 (Colberre-Garapin,
 et al., 1981, J. Mol. Biol. 150: 1); and hygromycin, which
 confers resistance to hygromycin (Santerre et al., 1984,
 25 Gene 30: 147). Additional selectable genes have been
 described, namely trpB, which allows cells to utilize
 indole in place of tryptophan; hisD, which allows cells
 to utilize histinol in place of histidine (Hartman &
 Mulligan, 1988, Proc. Natl. Acad. Sci. USA 85: 8047); and
 30 ODC (ornithine decarboxylase) which confers resistance to
 the ornithine decarboxylase inhibitor,
 2-(difluoromethyl)-DL-ornithine, DFMO (McConlogue, 1987,
 in Current Communications in Molecular Biology, Cold
 Spring Harbor Laboratory ed.).

35 In the third approach, transcriptional activity for
 the HBMYCNG coding region can be assessed by
 hybridization assays. For example, RNA can be isolated

and analyzed by Northern blot using a probe homologous to
5 the HBMYCNG coding sequence or particular portions
thereof. Alternatively, total nucleic acids of the host
cell may be extracted and assayed for hybridization to
such probes.

In the fourth approach, the expression of the
10 HBMYCNG protein product can be assessed immunologically,
for example by Western blots, immunoassays such as
radioimmuno-precipitation, enzyme-linked immunoassays and
the like. The ultimate test of the success of the
expression system, however, involves the detection of
15 biologically active HBMYCNG gene product. A number of
assays can be used to detect HBMYCNG activity including
but not limited to binding assays and biological assays
for HBMYCNG activity.

Once a clone that produces high levels of a
20 biologically active HBMYCNG polypeptide is identified,
the clone may be expanded and used to produce large
amounts of the polypeptide which may be purified using
techniques well known in the art, including but not
limited to, immunoaffinity purification using antibodies,
25 immunoprecipitation or chromatographic methods including
high performance liquid chromatography (HPLC).

Where the HBMYCNG coding sequence is engineered to
encode a cleavable fusion protein, purification may be
readily accomplished using affinity purification
30 techniques. For example, a collagenase cleavage
recognition consensus sequence may be engineered between
the carboxy terminus of HBMYCNG and protein A. The
resulting fusion protein may be readily purified using an
IgG column that binds the protein A moiety. Unfused
35 HBMYCNG may be readily released from the column by
treatment with collagenase. Another example would be the
use of pGEX vectors that express foreign polypeptides as

- fusion proteins with glutathione S-transferase (GST).
- 5 The fusion protein may be engineered with either thrombin or factor Xa cleavage sites between the cloned gene and the GST moiety. The fusion protein may be easily purified from cell extracts by adsorption to glutathione agarose beads followed by elution in the presence of glutathione.
 - 10 In fact, any cleavage site or enzyme cleavage substrate may be engineered between the HBMYCNG gene product sequence and a second peptide or protein that has a binding partner which could be used for purification, e.g., any antigen for which an immunoaffinity column can
 - 15 be prepared.

- In addition, HBMYCNG fusion proteins may be readily purified by utilizing an antibody specific for the fusion protein being expressed. For example, a system described by Janknecht et al. allows for the ready purification of
- 20 non-denatured fusion proteins expressed in human cell lines (Janknecht, et al., 1991, Proc. Natl. Acad. Sci. USA 88: 8972-8976). In this system, the gene of interest is subcloned into a vaccinia recombination plasmid such that the gene's open reading frame is translationally
 - 25 fused to an amino-terminal tag consisting of six histidine residues. Extracts from cells infected with recombinant vaccinia virus are loaded onto Ni^{2+} nitriloacetic acid-agarose columns and histidine-tagged proteins are selectively eluted with imidazole-containing
 - 30 buffers.

- In another embodiment, fusion proteins comprising at least one extracellular domain (i.e. the extracellular domains consist approximately of amino acid residues 161-173, 237-274, and 370-453) of the HBMYCNG polypeptide
- 35 are expressed from a genetically-engineered gene constructed and expressed using any recombinant method described above. In one aspect of this embodiment, a

"soluble" derivative of the HMBYCNG protein is
5 synthesized within which the six transmembrane domains
(represented by amino acid residues 141-160, 174-192,
217-236, 275-297, 350-369, and 454-474 of the protein
sequence of Figure 3) are replaced with peptide sequences
of comparable length and structure, providing a water
10 soluble fusion protein mimic of the HMBYCNG polypeptide.

Alternatively, the HMBYCNG protein itself can be
produced using chemical methods to synthesize the HMBYCNG
amino acid sequence in whole or in part. For example,
peptides can be synthesized by solid phase techniques,
15 cleaved from the resin, and purified by preparative high
performance liquid chromatography (see, e.g., Creighton,
1983, Proteins Structures And Molecular Principles, W.H.
Freeman and Co., N.Y., pp. 50-60). The composition of the
synthetic peptides may be confirmed by amino acid
20 analysis or sequencing (e.g., the Edman degradation
procedure; see Creighton, 1983, Proteins, Structures and
Molecular Principles, W.H. Freeman and Co., N.Y., pp.
34-49).

The HMBYCNG proteins, polypeptides and peptide
25 fragments, mutated, truncated or deleted forms of HMBYCNG
and/or HMBYCNG fusion proteins can be prepared for
various uses, including but not limited to, the
generation of antibodies, as reagents in diagnostic
assays, the identification of other cellular gene
30 products involved in ion transport, as reagents in assays
for screening for compounds for use in the treatment of
ion channel disorders.

5.3. Antibodies to HMBYCNG Polypeptides

35 The present invention also includes antibodies
directed to the HMBYCNG polypeptides of this invention
and methods for the production of those antibodies,

including antibodies that specifically recognize one or
5 more HBMYCNG epitopes or epitopes of conserved variants
or peptide fragments of HBMYCNG, or antibodies which
recognize the extracellular domains of the CNG α -subunit
polypeptides, or which recognize HBMYCNG epitopes within
the water soluble fusion protein mimic of the HBMYCNG
10 polypeptide disclosed above.

Such antibodies may include, but are not limited to,
polyclonal antibodies, monoclonal antibodies (mAbs),
humanized or chimeric antibodies, single chain
antibodies, Fab fragments, F(ab')₂ fragments, fragments
15 produced by a Fab expression library, anti-idiotypic
(anti-Id) antibodies, and epitope-binding fragments of
any of the above. Such antibodies may be used, for
example, in the detection of a HBMYCNG protein or
polypeptide in an biological sample and may, therefore,
20 be utilized as part of a diagnostic or prognostic
technique whereby patients may be tested for abnormal
levels of HBMYCNG and/or for the presence of abnormal
forms of the protein. Such antibodies may also be
utilized in conjunction with, for example, compound
25 screening protocols for the evaluation of the effect of
test compounds on HBMYCNG levels and/or activity.
Additionally, such antibodies can be used in conjunction
with the gene therapy techniques described in Section
5.4, *infra*, to, for example, evaluate normal and/or
30 genetically-engineered HBMYCNG-expressing cells prior to
their introduction into the patient.

An isolated polypeptide or peptide of the invention
can be used as an immunogen to generate antibodies using
standard techniques for polyclonal and monoclonal
35 antibody preparation. The full-length polypeptide or a
functional domain of the polypeptide, either native or
denatured, can be used or, alternatively, the invention

provides antigenic polypeptides or peptides for use as
 5 immunogens. The antigenic peptide of a polypeptide of the
 invention comprises at least 8 (preferably 10, 15, 20, or
 30) amino acid residues of the amino acid sequence of SEQ
 ID NO: 2 or a variant thereof, and features an epitope of
 the polypeptide such that an antibody raised against the
 10 peptide forms a specific immune complex with the
 polypeptide, and alternatively with a native polypeptide.

Preferred epitopes encompassed by the antigenic
 peptide are regions that are located on the surface of
 the polypeptide, e.g., hydrophilic regions. In certain
 15 embodiments, the nucleic acid molecules of the invention
 are present as part of nucleic acid molecules comprising
 nucleic acid sequences that contain or encode
 heterologous (e.g., vector, expression vector, or fusion
 polypeptide) sequences. These nucleotides can then be
 20 used to express polypeptides which can be used as
 immunogens to generate an immune response, or more
 particularly, to generate polyclonal or monoclonal
 antibodies specific to the expressed polypeptide.

For the production of antibodies against HBMYCNG,
 25 various host animals may be immunized by injection with
 the protein or a portion thereof. Such host animals
 include rabbits, mice, rats, and baboons. Various
 adjuvants may be used to increase the immunological
 response, depending on the host species, including but
 30 not limited to, Freund's (complete and incomplete),
 mineral gels such as aluminum hydroxide, surface active
 substances such as lysolecithin, pluronic polyols,
 polyanions, peptides, oil emulsions, keyhole limpet
 hemocyanin, dinitrophenol, and potentially useful human
 35 adjuvants such as BCG (bacille Calmette-Guerin) and
Corynebacterium parvum.

Accordingly, another aspect of the invention
5 pertains to antibodies directed against a polypeptide of
the invention. The term "antibody" as used herein refers
to immunoglobulin molecules and immunologically active
portions of immunoglobulin molecules, i.e., molecules
that contain an antigen binding site which specifically
10 binds an antigen, such as a polypeptide of the invention,
e.g., an epitope of a polypeptide of the invention. A
molecule which specifically binds to a given polypeptide
of the invention is a molecule which binds the
polypeptide, but does not substantially bind other
15 molecules in a sample, e.g., a biological sample, which
naturally contains the polypeptide. Examples of
immunologically active portions of immunoglobulin
molecules include F(ab) and F(ab')₂ fragments which can be
generated by treating the antibody with an enzyme such as
20 pepsin. The invention provides polyclonal and monoclonal
antibodies. The term "monoclonal antibody" or "monoclonal
antibody composition," as used herein, refers to a
population of antibody molecules that contain only one
species of an antigen binding site capable of
25 immunoreacting with a particular epitope.

Polyclonal antibodies are heterogeneous populations
of antibody molecules derived from the sera of animals
immunized with an antigen, such as a HBMYCNG polypeptide,
or an antigenic functional derivative thereof. For the
30 production of polyclonal antibodies, host animals such as
those described above, may be immunized by injection with
the HBMYCNG polypeptide supplemented with adjuvants as
also described above.

Monoclonal antibodies, which are homogeneous
35 populations of antibodies to a particular antigen, may be
obtained by any technique which provides for the
production of antibody molecules by continuous cell lines

in culture. These include, but are not limited to, the
 5 hybridoma technique of Kohler and Milstein (1975, Nature
 256: 495-497; and U.S. Patent No. 4,376,110), the human
 B-cell hybridoma technique (Kosbor et al., 1983,
 Immunology Today 4: 72; Cole et al., 1983, Proc. Natl.
 Acad. Sci. USA 80: 2026-2030), and the EBV-hybridoma
 10 technique (Cole et al., 1985, Monoclonal Antibodies And
 Cancer Therapy, Alan R. Liss, Inc., pp. 77-96). Such
 antibodies may be of any immunoglobulin class including
 IgG, IgM, IgE, IgA, IgD and any subclass thereof. The
 hybridomas producing the monoclonal antibodies of this
 15 invention may be cultivated *in vitro* or *in vivo*.

In addition, techniques developed for the production
 of chimeric antibodies (Morrison et al., 1984, Proc.
 Natl. Acad. Sci., 81: 6851-6855; Neuberger et al., 1984,
 Nature 312: 604-608; Takeda et al., 1985, Nature 314:
 20 452-454) by splicing the genes from a mouse antibody
 molecule of appropriate antigen specificity together with
 genes from a human antibody molecule of appropriate
 biological activity can be used. A chimeric antibody is a
 molecule in which different portions are derived from
 25 different animal species, such as those having a variable
 region derived from a murine mAb and a human
 immunoglobulin constant region (see, e.g., Cabilly et
 al., U.S. Patent No. 4,816,567; and Boss et al., U.S.
 Patent No. 4,816,397.)

30 The antibody titer in the immunized subject can be
 monitored over time by standard techniques, such as with
 an enzyme linked immunosorbent assay (ELISA) using
 immobilized polypeptide. If desired, the antibody
 molecules can be isolated from the mammal (e.g., from the
 35 blood) and further purified by well-known techniques,
 such as protein A chromatography to obtain the IgG
 fraction. Alternatively, antibodies specific for a

polypeptide or peptide of the invention can be selected
5 for (e.g., partially purified) or purified by, e.g.,
affinity chromatography. For example, a recombinantly
expressed and purified (or partially purified)
polypeptide of the invention is produced as described
herein, and covalently or non-covalently coupled to a
10 solid support such as, for example, a chromatography
column. The column can then be used to affinity purify
antibodies specific for the polypeptides of the invention
from a sample containing antibodies directed against a
large number of different epitopes, thereby generating a
15 substantially purified antibody composition, i.e., one
that is substantially free of contaminating antibodies.
By a substantially purified antibody composition is
meant, in this context, that the antibody sample contains
at most only 30% (by dry weight) of contaminating
20 antibodies directed against epitopes other than those on
the desired polypeptide or polypeptide of the invention,
and preferably at most 20%, yet more preferably at most
10%, and most preferably at most 5% (by dry weight) of
the sample is contaminating antibodies. A purified
25 antibody composition means that at least 99% of the
antibodies in the composition are directed against the
desired polypeptide or peptide of the invention.

At an appropriate time after immunization, e.g.,
when the specific antibody titers are highest,
30 antibody-producing cells can be obtained from the subject
and used to prepare monoclonal antibodies by standard
techniques, such as the hybridoma technique originally
described by Kohler and Milstein (1975) Nature
256:495-497, the human B cell hybridoma technique (Kozbor
35 et al. (1983) Immunol. Today 4:72), the EBV-hybridoma
technique (Cole et al. (1985), Monoclonal Antibodies and
Cancer Therapy, Alan R. Liss, Inc., pp. 77-96) or trioma

techniques. The technology for producing hybridomas is
 5 well known (see generally Current Protocols in Immunology
 (1994) Coligan et al. (eds.) John Wiley & Sons, Inc., New
 York, NY). Hybridoma cells producing a monoclonal
 antibody of the invention are detected by screening the
 hybridoma culture supernatants for antibodies that bind
 10 the polypeptide of interest, e.g., using a standard ELISA
 assay.

Alternative to preparing monoclonal antibody-
 secreting hybridomas, a monoclonal antibody directed
 against a polypeptide of the invention can be identified
 15 and isolated by screening a recombinant combinatorial
 immunoglobulin library (e.g., an antibody phage display
 library) with the polypeptide of interest. Kits for
 generating and screening phage display libraries are
 commercially available (e.g., the Pharmacia Recombinant
 20 Phage Antibody System, Catalog No. 27-9400-01; and the
 Stratagene SurfZAP Phage Display Kit, Catalog No.
 240612). Additionally, examples of methods and reagents
 particularly amenable for use in generating and screening
 antibody display library can be found in, for example,
 25 U.S. Patent No. 5,223,409; PCT Publication No. WO
 92/18619; PCT Publication No. WO 91/17271; PCT
 Publication No. WO 92/20791; PCT Publication No. WO
 92/15679; PCT Publication No. WO 93/01288; PCT
 Publication No. WO 92/01047; PCT Publication No. WO
 30 92/09690; PCT Publication No. WO 90/02809; Fuchs et al.
 (1991) Bio/Technology 9:1370-1372; Hay et al. (1992) Hum.
 Antibod. Hybridomas 3:81-85; Huse et al. (1989) Science
 246:1275-1281; Griffiths et al. (1993) EMBO J.
 12:725-734.

35 In addition, techniques have been developed for the
 production of humanized antibodies (see, e.g., Queen,
 U.S. Patent No. 5,585,089). Humanized antibodies are

antibody molecules from non-human species having one or
5 more CDRs from the non-human species and a framework
region from a human immunoglobulin molecule.

Completely human antibodies are particularly
desirable for therapeutic treatment of human patients.
Such antibodies can be produced, for example, using
10 transgenic mice which are incapable of expressing
endogenous immunoglobulin heavy and light chains genes,
but which can express human heavy and light chain genes.
The transgenic mice are immunized in the normal fashion
with a selected antigen, e.g., all or a portion of a
15 polypeptide of the invention. Monoclonal antibodies
directed against the antigen can be obtained using
conventional hybridoma technology. The human
immunoglobulin transgenes harbored by the transgenic mice
rearrange during B cell differentiation, and subsequently
20 undergo class switching and somatic mutation. Thus, using
such a technique, it is possible to produce
therapeutically useful IgG, IgA and IgE antibodies. For
an overview of this technology for producing human
antibodies, see Lonberg and Huszar (1995, Int. Rev.
25 Immunol. 13:65-93). For a detailed discussion of this
technology for producing human antibodies and human
monoclonal antibodies and protocols for producing such
antibodies, see, e.g., U.S. Patent 5,625,126; U.S. Patent
5,633,425; U.S. Patent 5,569,825; U.S. Patent 5,661,016;
30 and U.S. Patent 5,545,806. In addition, companies such as
Abgenix, Inc. (Freemont, CA), can be engaged to provide
human antibodies directed against a selected antigen
using technology similar to that described above.

Completely human antibodies which recognize a
35 selected epitope can be generated using a technique
referred to as "guided selection." In this approach a
selected non-human monoclonal antibody, e.g., a mouse

antibody, is used to guide the selection of a completely
5 human antibody recognizing the same epitope (Jespers et
al. (1994) Bio/technology 12:899-903).

Alternatively, techniques described for the
production of single chain antibodies (U.S. Patent
4,946,778; Bird, 1988, Science 242: 423-426; Huston et
10 al., 1988, Proc. Natl. Acad. Sci. USA 85: 5879-5883; and
Ward et al., 1989, Nature 334: 544-546) can be used in
the production of single chain antibodies against
HBMYCNG. Single chain antibodies are formed by linking
the heavy and light chain fragments of the Fv region via
15 an amino acid bridge, resulting in a single chain
polypeptide.

Furthermore, antibody fragments which recognize
specific epitopes of HBMYCNG may be produced by
techniques well known in the art. For example, such
20 fragments include but are not limited to, $F(ab')_2$
fragments which can be produced by pepsin digestion of
the antibody molecule and Fab fragments which can be
generated by reducing the disulfide bridges of the $F(ab')_2$
fragments. Alternatively, Fab expression libraries may be
25 constructed (Huse et al., 1989, Science 246: 1275-1281)
to allow rapid and easy identification of monoclonal Fab
fragments with the desired specificity.

An antibody directed against a polypeptide of the
invention (e.g., monoclonal antibody) can be used to
30 isolate the polypeptide by standard techniques, such as
affinity chromatography or immunoprecipitation. Moreover,
such an antibody can be used to detect the polypeptide
(e.g., in a cellular lysate or cell supernatant) in order
to evaluate the abundance and pattern of expression of
35 the polypeptide. The antibodies can also be used
diagnostically to monitor polypeptide levels in tissue as
part of a clinical testing procedure, e.g., to, for

- example, determine the efficacy of a given treatment
- 5 regimen. Detection can be facilitated by coupling the antibody to a detectable substance. Examples of detectable substances include various enzymes, prosthetic groups, fluorescent materials, luminescent materials, bioluminescent materials, and radioactive materials.
- 10 Examples of suitable enzymes include horseradish peroxidase, alkaline phosphatase, β -galactosidase, or acetylcholinesterase; examples of suitable prosthetic group complexes include streptavidin/biotin and avidin/biotin; examples of suitable fluorescent materials
- 15 include umbelliferone, fluorescein, fluorescein isothiocyanate, rhodamine, dichlorotriazinylamine fluorescein, dansyl chloride or phycoerythrin; an example of a luminescent material includes luminol; examples of bioluminescent materials include luciferase, luciferin,
- 20 and aequorin, and examples of suitable radioactive material include ^{125}I , ^{131}I , ^{35}S , ^{99}Tc or ^3H .

In addition, the HBMYCNG gene sequences and gene products, including polypeptides, peptides, fusion polypeptides or peptides, and antibodies directed against

25 said gene products and peptides, have applications for purposes independent of the role of the gene products. For example, HBMYCNG gene products, including polypeptides or peptides, as well as specific antibodies thereto, can be used for construction of fusion

30 polypeptides to facilitate recovery, detection, or localization of another polypeptide of interest. In addition, HBMYCNG genes and gene products can be used for genetic mapping. Finally, HBMYCNG nucleic acids and gene products have generic uses, such as supplemental sources

35 of nucleic acids, polypeptides and amino acids for food additives or cosmetic products.

Further, an antibody of the invention (or fragment thereof) may be conjugated to a therapeutic moiety such as a cytotoxin, a therapeutic agent or a radioactive metal ion. A cytotoxin or cytotoxic agent includes any agent that is detrimental to cells. Examples include taxol, cytochalasin B, gramicidin D, ethidium bromide, emetine, mitomycin, etoposide, tenoposide, vincristine, vinblastine, colchicin, doxorubicin, daunorubicin, dihydroxy anthracin dione, mitoxantrone, mithramycin, actinomycin D, 1-dehydrotestosterone, glucocorticoids, procaine, tetracaine, lidocaine, propranolol, and puromycin and analogs or homologs thereof. Therapeutic agents include, but are not limited to, antimetabolites (e.g., methotrexate, 6-mercaptopurine, 6-thioguanine, cytarabine, 5-fluorouracil decarbazine), alkylating agents (e.g., mechlorethamine, thioepa chlorambucil, melphalan, carmustine (BSNU) and lomustine (CCNU), cyclophosphamide, busulfan, dibromomannitol, streptozotocin, mitomycin C, and cis-dichlorodiamine platinum (II) (DDP) cisplatin), anthracyclines (e.g., daunorubicin (formerly daunomycin) and doxorubicin), antibiotics (e.g., dactinomycin (formerly actinomycin), bleomycin, mithramycin, and anthramycin (AMC)), and anti-mitotic agents (e.g., vincristine and vinblastine).

In addition, polypeptides, agonists or antagonists which bind a polypeptide of the invention can also be conjugated to the foregoing, thereby targeting a toxin to cells expressing HGPRBMY1.

The conjugates of the invention can be used for modifying a given biological response, the drug moiety is not to be construed as limited to classical chemical therapeutic agents. For example, the drug moiety may be a polypeptide or peptide possessing a desired biological activity. Such polypeptides may include, for example, a

toxin such as abrin, ricin A, pseudomonas exotoxin, or
 5 diphtheria toxin; a polypeptide such as tumor necrosis
 factor, γ -interferon, α -interferon, nerve growth factor,
 platelet derived growth factor, tissue plasminogen
 activator, a thrombotic agent or an anti-angiogenic
 agent, e.g., angiostatin or endostatin; or, biological
 10 response modifiers such as, for example, lymphokines,
 interleukin-1 ("IL-1"), interleukin-2 ("IL-2"),
 interleukin-4 ("IL-4"), interleukin-6 ("IL-6"),
 interleukin-7 ("IL-7"), granulocyte macrophage colony
 stimulating factor ("GM-CSF"), granulocyte colony
 15 stimulating factor ("G-CSF"), interleukin-10 ("IL-10"),
 interleukin-12 ("IL-12"), interleukin-17 ("IL-15"),
 interleukin-17 ("IL-17"), interferon- γ ("IFN- γ "),
 interferon- α ("IFN- α "), or other immune factors or growth
 factors.

20 Techniques for conjugating such therapeutic moiety
 to antibodies are well known, see, e.g., Arnon et al.,
 "Monoclonal Antibodies For Immunotargeting Of Drugs In
 Cancer Therapy", in Monoclonal Antibodies And Cancer
 Therapy, Reisfeld et al. (eds.), pp. 243-56 (Alan R.
 25 Liss, Inc. 1985); Hellstrom et al., "Antibodies For Drug
 Delivery", in Controlled Drug Delivery (2nd Ed.),
 Robinson et al. (eds.), pp. 623-53 (Marcel Dekker, Inc.
 1987); Thorpe, "Antibody Carriers Of Cytotoxic Agents In
 Cancer Therapy: A Review", in Monoclonal Antibodies:
 30 Biological And Clinical Applications, Pinchera et al.
 (eds.), pp. 475-506 (1985); "Analysis, Results, And
 Future Prospective Of The Therapeutic Use Of Radiolabeled
 Antibody In Cancer Therapy", in Monoclonal Antibodies For
 Cancer Detection And Therapy, Baldwin et al. (eds.), pp.
 35 303-16 (Academic Press 1985), and Thorpe et al., "The
 Preparation And Cytotoxic Properties Of Antibody-Toxin
 Conjugates", Immunol. Rev., 62:119-58 (1982).

5 An antibody with or without a therapeutic moiety conjugated to it can be used as a therapeutic that is administered alone or in combination with chemotherapeutic agents.

Alternatively, an antibody of the invention can be conjugated to a second antibody to form an "antibody heteroconjugate" as described by Segal in U.S. Patent No. 10 4,676,980 or alternatively, the antibodies can be conjugated to form an "antibody heteropolymer" as described in Taylor et al., in U.S. Patent Nos. 5,470,570 and 5,487,890.

15 An antibody with or without a therapeutic moiety conjugated to it can be used as a therapeutic that is administered alone or in combination with cytotoxic factor(s) and/or cytokine(s).

In yet a further aspect, the invention provides 20 substantially purified antibodies or fragments thereof, including human or non-human antibodies or fragments thereof, which antibodies or fragments specifically bind to a polypeptide of the invention comprising an amino acid sequence of SEQ ID NO: 2 or a variant thereof. In 25 various embodiments, the substantially purified antibodies of the invention, or fragments thereof, can be human, non-human, chimeric and/or humanized antibodies.

In another aspect, the invention provides human or non-human antibodies or fragments thereof, which 30 antibodies or fragments specifically bind to a polypeptide comprising an amino acid sequence of SEQ ID NO: 2 or a variant thereof. Such non-human antibodies can be goat, mouse, sheep, horse, chicken, rabbit, or rat antibodies. Alternatively, the non-human antibodies of 35 the invention can be chimeric and/or humanized antibodies. In addition, the non-human antibodies of the

invention can be polyclonal antibodies or monoclonal
5 antibodies.

In still a further aspect, the invention provides
monoclonal antibodies or fragments thereof, which
antibodies or fragments specifically bind to a
polypeptide of the invention comprising an amino acid
10 sequence of SEQ ID NO: 2 or a variant thereof. The
monoclonal antibodies can be human, humanized, chimeric
and/or non-human antibodies.

The substantially purified antibodies or fragments
thereof specifically bind to a signal peptide, a secreted
15 sequence, an extracellular domain, a transmembrane or a
cytoplasmic domain of a polypeptide of the invention. In
a particularly preferred embodiment, the substantially
purified antibodies or fragments thereof, the non-human
antibodies or fragments thereof, and/or the monoclonal
20 antibodies or fragments thereof, of the invention
specifically bind to a secreted sequence, or
alternatively, to an extracellular domain of the amino
acid sequence of the invention.

Any of the antibodies of the invention can be
25 conjugated to a therapeutic moiety or to a detectable
substance. Non-limiting examples of detectable substances
that can be conjugated to the antibodies of the invention
are an enzyme, a prosthetic group, a fluorescent
material, a luminescent material, a bioluminescent
30 material, and a radioactive material.

The invention also provides a kit containing an
antibody of the invention conjugated to a detectable
substance, and instructions for use. Still another aspect
of the invention is a pharmaceutical composition
35 comprising an antibody of the invention and a
pharmaceutically acceptable carrier. In preferred
embodiments, the pharmaceutical composition contains an

antibody of the invention, a therapeutic moiety, and a
 5 pharmaceutically acceptable carrier.

Still another aspect of the invention is a method of
 making an antibody that specifically recognizes HBMYCNG,
 the method comprising immunizing a mammal with a
 polypeptide. After immunization, a sample is collected
 10 from the mammal that contains an antibody that
 specifically recognizes the immunogen. Preferably, the
 polypeptide is recombinantly produced using a non-human
 host cell. Optionally, the antibodies can be further
 purified from the sample using techniques well known to
 15 those of skill in the art. The method can further
 comprise producing a monoclonal antibody-producing cell
 from the cells of the mammal. Optionally, antibodies are
 collected from the antibody-producing cell.

20 5.4. Uses of the HBMYCNG Nucleic Acid Molecules, Gene Products, and Antibodies

As discussed *supra*, the HBMYCNG gene of this
 invention encodes a protein involved in the formation or
 function of ion channels, more particularly, cation
 25 channels. Given the importance of cations such as
 calcium, sodium or potassium in many cellular processes,
 the HBMYCNG nucleic acid molecules and polypeptides of
 this invention are useful for the diagnosis and treatment
 of a variety of human disease conditions which involve
 30 ion, more particularly, cation, channel dysfunction.

For example, calcium plays a role in the release of
 neurotransmitters, hormones and other circulating
 factors, the expression of numerous regulatory genes as
 well as the cellular process of apoptosis or cell death.
 35 Potassium provides for neuroprotection and also affects
 insulin secretion. Sodium is involved in the regulation
 of normal neuronal action potential generation and

propagation. Sodium channel blockers such as lidocaine
5 are important analgesics. Therefore, cation channel
dysfunction may play a role in many human diseases and
disorders such as CNS disorders, e.g., stroke, anxiety,
and depression, Alzheimer's disease, or Parkinson's
10 e.g., arrhythmia, diabetes, chronic pain, hypercalcemia,
hypercalciuria, or ion channel dysfunction that is
associated with immunological disorders,
gastro-intestinal (GI) tract disorders, or renal or liver
15 play a role in the proper functioning of the serotonin
nervous system which also participates in the control of
anxiety, fear, depression, sleep and pain. Accordingly,
cation channel dysfunction may further play a role in
anxiety and fear disorders, bipolar and major depression,
20 panic disorder, headaches, migraine, disorders of
circadian rhythmicity, stress, various sexual
dysfunctions including but not limited to erectile
dysfunction, neuroleptic-induced catalepsy, Rett syndrome
and aggressive behaviors. As such, proteins that are
25 involved in either the formation or function of these ion
channels (and the nucleic acids that encode those
proteins) are useful for the diagnosis and treatment of
many human diseases.

Among the uses for the nucleic acid molecules and
30 polypeptides of the invention are the prognostic and
diagnostic evaluation of human disorders involving
ion/cation channel dysfunction, and the identification of
subjects with a predisposition to such disorders, as
described below. Other uses include methods for the
35 treatment of such ion/cation channel dysfunction
disorders, for the modulation of HBMYCNG gene-mediated

activity, and for the modulation of HBMYCNG-mediated
5 effector functions.

In addition, the nucleic acid molecules and
polypeptides of the invention can be used in assays for
the identification of compounds which modulate the
expression of the HBMYCNG genes of the invention and/or
10 the activity of the HBMYCNG gene products. Such compounds
can include, for example, other cellular products or
small molecule compounds that are involved in cation
homeostasis or activity.

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5 5.4.1. Diagnosis and Prognosis of Ion-Related Disorders

Methods of the invention for the diagnosis and prognosis of human diseases involving ion, e.g., cation, dysfunction may utilize reagents such as the HBMYCNG nucleic acid molecules and sequences described in Sections 5.1, *supra*, or antibodies directed against HBMYCNG polypeptides, including peptide fragments thereof, as described in Section 5.3., *supra*. Specifically, such reagents may be used, for example, for: (1) the detection of the presence of HBMYCNG gene mutations, or the detection of either over- or under-expression of HBMYCNG gene mRNA relative to the non-cation dysfunctional state or the qualitative or quantitative detection of alternatively spliced forms of HBMYCNG transcripts which may correlate with certain ion homeostasis disorders or susceptibility toward such disorders; and (2) the detection of either an over- or an under-abundance of HBMYCNG gene product relative to the non- cation dysfunctional state or the presence of a modified (e.g., less than full length) HBMYCNG gene product which correlates with a cation dysfunctional state or a progression toward such a state.

The methods described herein may be performed, for example, by utilizing pre-packaged diagnostic test kits comprising at least one specific HBMYCNG gene nucleic acid or anti-HBMYCNG gene antibody reagent described herein, which may be conveniently used, e.g., in clinical settings, to screen and diagnose patients exhibiting ion/cation channel/homeostasis abnormalities and to screen and identify those individuals exhibiting a predisposition to such abnormalities.

For the detection of HBMYCNG mutations, any
 5 nucleated cell can be used as a starting source for
 genomic nucleic acid. For the detection of HBMYCNG
 transcripts or HBMYCNG gene products, any cell type or
 tissue in which the HBMYCNG gene is expressed may be
 utilized.

10 Nucleic acid-based detection techniques are
 described in Section 5.4.1.1., *infra*, whereas
 peptide-based detection techniques are described in
 Section 5.4.1.2., *infra*.

15 5.4.1.1. Detection of Hbmycng Gene Nucleic Acid
Molecules

Mutations or polymorphisms within the HBMYCNG gene
 can be detected by utilizing a number of techniques.
 Nucleic acid from any nucleated cell can be used as the
 20 starting point for such assay techniques, and may be
 isolated according to standard nucleic acid preparation
 procedures which are well known to those of skill in the
 art.

Genomic DNA may be used in hybridization or
 25 amplification assays of biological samples to detect
 abnormalities involving HBMYCNG gene structure, including
 point mutations, insertions, deletions and chromosomal
 rearrangements. Such assays may include, but are not
 limited to, direct sequencing (Wong, C. et al., 1987,
 30 Nature 330:384-386), single stranded conformational
 polymorphism analyses (SSCP; Orita, M. et al., 1989,
 Proc. Natl. Acad. Sci. USA 86:2766-2770), heteroduplex
 analysis (Keen, T.J. et al., 1991, Genomics 11:199-205;
 Perry, D.J. & Carrell, R.W., 1992), denaturing gradient
 35 gel electrophoresis (DGGE; Myers, R.M. et al., 1985,
 Nucl. Acids Res. 13:3131-3145), chemical mismatch
 cleavage (Cotton, R.G. et al., 1988, Proc. Natl. Acad.

- Sci. USA 85:4397-4401) and oligonucleotide hybridization
5 (Wallace, R.B. et al., 1981, Nucl. Acids Res. 9:879-894;
Lipshutz, R.J. et al., 1995, Biotechniques 19:442-447).

Diagnostic methods for the detection of HBMYCNG gene specific nucleic acid molecules, in patient samples or other appropriate cell sources, may involve the
10 amplification of specific gene sequences, e.g., by PCR, followed by the analysis of the amplified molecules using techniques well known to those of skill in the art, such as, for example, those listed above. Utilizing analysis techniques such as these, the amplified sequences can be
15 compared to those which would be expected if the nucleic acid being amplified contained only normal copies of the HBMYCNG gene in order to determine whether a HBMYCNG gene mutation exists.

Further, well-known genotyping techniques can be
20 performed to type polymorphisms that are in close proximity to mutations in the HBMYCNG gene itself. These polymorphisms can be used to identify individuals in families likely to carry mutations. If a polymorphism exhibits linkage disequilibrium with mutations in the
25 HBMYCNG gene, it can also be used to identify individuals in the general population likely to carry mutations. Polymorphisms that can be used in this way include restriction fragment length polymorphisms (RFLPs), which involve sequence variations in restriction enzyme target
30 sequences, single-base polymorphisms and simple sequence repeat polymorphisms (SSLPs).

For example, Weber (U.S. Pat. No. 5,075,217) describes a DNA marker based on length polymorphisms in blocks of (dC-dA)_n-(dG-dT)_n short tandem repeats. The
35 average separation of (dC-dA)_n-(dG-dT)_n blocks is estimated to be 30,000-60,000 bp. Markers which are so closely spaced exhibit a high frequency co-inheritance,

and are extremely useful in the identification of genetic mutations, such as, for example, mutations within the HBMYCNG gene, and the diagnosis of diseases and disorders related to HBMYCNG mutations.

Also, Caskey et al. (U.S. Pat.No. 5,364,759) describe a DNA profiling assay for detecting short tri- and tetra- nucleotide repeat sequences. The process includes extracting the DNA of interest, such as the HBMYCNG gene, amplifying the extracted DNA, and labelling the repeat sequences to form a genotypic map of the individual's DNA.

A HBMYCNG probe could additionally be used to directly identify RFLPs. Additionally, a HBMYCNG probe or primers derived from the HBMYCNG sequences of the invention could be used to isolate genomic clones such as YACs, BACs, PACs, cosmids, phage or plasmids. The DNA contained in these clones can be screened for single-base polymorphisms or simple sequence length polymorphisms (SSLPs) using standard hybridization or sequencing procedures.

Alternative diagnostic methods for the detection of HBMYCNG gene-specific mutations or polymorphisms can include hybridization techniques which involve for example, contacting and incubating nucleic acids including recombinant DNA molecules, cloned genes or degenerate variants thereof, obtained from a sample, e.g., derived from a patient sample or other appropriate cellular source, with one or more labeled nucleic acid reagents including the HBMYCNG nucleic acid molecules of the invention including recombinant DNA molecules, cloned genes or degenerate variants thereof, as described in Section 5.1 *supra*, under conditions favorable for the specific annealing of these reagents to their complementary sequences within the HBMYCNG gene.

Preferably, the lengths of these nucleic acid reagents
5 are at least 15 to 30 nucleotides. After incubation, all
non-annealed nucleic acids are removed from the nucleic
acid:HBMYCNG molecule hybrid. The presence of nucleic
acids which have hybridized, if any such molecules exist,
is then detected. Using such a detection scheme, the
10 nucleic acid from the cell type or tissue of interest can
be immobilized, for example, to a solid support such as a
membrane, or a plastic surface such as that on a
microtiter plate or polystyrene beads. In this case,
after incubation, non-annealed, labeled nucleic acid
15 molecules of the invention as described in Section 5.1
are easily removed. Detection of the remaining, annealed,
labeled HBMYCNG nucleic acid reagents is accomplished
using standard techniques well-known to those in the art.
The HBMYCNG gene sequences to which the nucleic acid
20 molecules of the invention have annealed can be compared
to the annealing pattern expected from a normal HBMYCNG
gene sequence in order to determine whether a HBMYCNG
gene mutation is present.

Quantitative and qualitative aspects of HBMYCNG gene
25 expression can also be assayed. For example, RNA from a
cell type or tissue known, or suspected, to express the
HBMYCNG gene may be isolated and tested utilizing
hybridization or PCR techniques as described *supra*. The
isolated cells can be derived from cell culture or from a
30 patient. The analysis of cells taken from culture may be
a necessary step in the assessment of cells to be used as
part of a cell-based gene therapy technique or,
alternatively, to test the effect of compounds on the
expression of the HBMYCNG gene. Such analyses may reveal
35 both quantitative and qualitative aspects of the
expression pattern of the HBMYCNG gene, including

activation or inactivation of HBMYCNG gene expression and
5 presence of alternatively spliced HBMYCNG transcripts.

In one embodiment of such a detection scheme, a cDNA
molecule is synthesized from an RNA molecule of interest
(e.g., by reverse transcription of the RNA molecule into
cDNA). All or part of the resulting cDNA is then used as
10 the template for a nucleic acid amplification reaction,
such as a PCR amplification reaction, or the like. The
nucleic acid reagents used as synthesis initiation
reagents (e.g., primers) in the reverse transcription and
nucleic acid amplification steps of this method are
15 chosen from among the HBMYCNG nucleic acid molecules of
the invention as described in Section 5.1, *supra*. The
preferred lengths of such nucleic acid reagents are at
least 9-30 nucleotides.

For detection of the amplified product, the nucleic
20 acid amplification may be performed using radioactively
or non-radioactively labeled nucleotides. Alternatively,
enough amplified product may be made such that the
product may be visualized by standard ethidium bromide
staining or by utilizing any other suitable nucleic acid
25 staining method.

Such RT-PCR techniques can be utilized to detect
differences in HBMYCNG transcript size which may be due
to normal or abnormal alternative splicing. Additionally,
such techniques can be utilized to detect quantitative
30 differences between levels of full length and/or
alternatively spliced HBMYCNG transcripts detected in
normal individuals relative to those individuals
exhibiting ion dysfunction disorders or exhibiting a
predisposition to toward such disorders.

35 In the case where detection of specific
alternatively spliced species is desired, appropriate
primers and/or hybridization probes can be used, such

that, in the absence of such sequence, no amplification
 5 would occur. Alternatively, primer pairs may be chosen
 utilizing the sequences depicted in FIG. 1, 3 or 5 to
 choose primers which will yield fragments of differing
 size depending on whether a particular exon is present or
 absent from the HBMYCNG transcript being utilized.

10 As an alternative to amplification techniques,
 standard Northern analyses can be performed if a
 sufficient quantity of the appropriate cells can be
 obtained. Utilizing such techniques, quantitative as well
 as size-related differences between HBMYCNG transcripts
 15 can also be detected.

Additionally, it is possible to perform HBMYCNG gene
 expression assays *in situ*, i.e., directly upon tissue
 sections (fixed and/or frozen) of patient tissue obtained
 from biopsies or resections, such that no nucleic acid
 20 purification is necessary. The nucleic acid molecules of
 the invention as described in Section 5.1 may be used as
 probes and/or primers for such *in situ* procedures (see,
 for example, Nuovo, G.J., 1992, "PCR In Situ
 Hybridization: Protocols And Applications", Raven Press,
 25 NY).

5.4.1.2. Detection of HBMYCNG Gene Products

Antibodies directed against wild type or mutant
 HBMYCNG gene products or conserved variants or peptide
 30 fragments or extracellular domain thereof as described
supra may also be used for the diagnosis and prognosis of
 ion or cation-related disorders. Such diagnostic methods
 may be used to detect abnormalities in the level of
 HBMYCNG gene expression or abnormalities in the structure
 35 and/or temporal, tissue, cellular, or subcellular
 location of HBMYCNG gene products. Antibodies, or
 fragments of antibodies, may be used to screen

5 potentially therapeutic compounds *in vitro* to determine their effects on HBMYCNG gene expression and HBMYCNG peptide production. The compounds which have beneficial effects on ion and cation-related disorders can be identified and a therapeutically effective dose determined.

10 *In vitro* immunoassays may be used, for example, to assess the efficacy of cell-based gene therapy for ion or cation-related disorders. For example, antibodies directed against HBMYCNG peptides may be used *in vitro* to determine the level of HBMYCNG gene expression achieved
15 in cells genetically engineered to produce HBMYCNG peptides. Such analysis will allow for a determination of the number of transformed cells necessary to achieve therapeutic efficacy *in vivo*, as well as optimization of the gene replacement protocol.

20 The tissue or cell type to be analyzed will generally include those which are known, or suspected, to express the HBMYCNG gene. The protein isolation methods employed may, for example, be such as those described in Harlow, E. and Lane, D., 1988, "Antibodies: A Laboratory
25 Manual", Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York. The isolated cells can be derived from cell culture or from a patient. The analysis of cells taken from culture may be a necessary step in the assessment of cells to be used as part of a cell-based
30 gene therapy technique or, alternatively, to test the effect of compounds on the expression of the HBMYCNG gene.

Preferred diagnostic methods for the detection of HBMYCNG gene products or conserved variants or peptide
35 fragments thereof, may involve, for example, immunoassays wherein the HBMYCNG gene products or conserved variants, including gene products which are the result of

alternatively spliced transcripts, or peptide fragments
5 are detected by their interaction with an anti-HBMYCNG
gene product-specific antibody.

For example, antibodies, or fragments of antibodies,
such as those described in Section 5.3 *supra*, may be used
to quantitatively or qualitatively detect the presence of
10 HBMYCNG gene products or conserved variants or peptide
fragments thereof. The antibodies (or fragments thereof)
may, additionally, be employed histologically, as in
immunofluorescence or immunoelectron microscopy, for *in*
situ detection of HBMYCNG gene products or conserved
15 variants or peptide fragments thereof. *In situ* detection
may be accomplished by removing a histological specimen
from a patient, and applying thereto a labeled HBMYCNG
antibody of the present invention. The antibody (or
fragment) is preferably applied by overlaying the labeled
20 antibody (or fragment) onto a biological sample. Through
the use of such a procedure, it is possible to determine
not only the presence of the HBMYCNG gene product, or
conserved variants or peptide fragments, but also its
distribution in the examined tissue. Using the present
25 invention, those of ordinary skill will readily perceive
that any of a wide variety of histological methods (such
as staining procedures) can be modified in order to
achieve such *in situ* detection.

Immunoassays for HBMYCNG gene products or conserved
30 variants or peptide fragments thereof will typically
comprise incubating a sample, such as a biological fluid,
a tissue extract, freshly harvested cells, or lysates of
cells which have been incubated in cell culture, in the
presence of a detectably labeled antibody capable of
35 identifying HBMYCNG gene products or conserved variants
or peptide fragments thereof, and detecting the bound

antibody by any of a number of techniques well-known in
5 the art.

The biological sample may be brought in contact with
and immobilized onto a solid phase support or carrier
such as nitrocellulose, or other solid support which is
capable of immobilizing cells, cell particles or soluble
10 proteins. The support may then be washed with suitable
buffers followed by treatment with the detectably labeled
HBMYCNG gene specific antibody. The solid phase support
may then be washed with the buffer a second time to
remove unbound antibody. The amount of bound label on
15 solid support may then be detected by conventional means.

By "solid phase support or carrier" is intended any
support capable of binding an antigen or an antibody.
Well-known supports or carriers include glass,
polystyrene, polypropylene, polyethylene, dextran, nylon,
20 amylases, natural and modified celluloses,
polyacrylamides, gabbros, and magnetite. The nature of
the carrier can be either soluble to some extent or
insoluble. The support material may have virtually any
possible structural configuration so long as the coupled
25 molecule is capable of binding to an antigen or antibody.
Thus, the support configuration may be spherical, as in a
bead, or cylindrical, as in the inside surface of a test
tube, or the external surface of a rod. Alternatively,
the surface may be flat such as a sheet, test strip, etc.
30 Preferred supports include polystyrene beads. Those
skilled in the art will know many other suitable carriers
for binding antibody or antigen, or will be able to
ascertain the same by use of routine experimentation.

The binding activity of a given lot of anti-HBMYCNG
35 gene product antibody may be determined according to well
known methods. Those skilled in the art will be able to

determine operative and optimal assay conditions for each
 5 determination by employing routine experimentation.

One of the ways in which the HBMYCNG gene
 peptide-specific antibody can be detectably labeled is by
 linking the antibody to an enzyme in an enzyme
 immunoassay (EIA) (Voller, A., "The Enzyme Linked
 10 Immunosorbent Assay (ELISA)", 1978, Diagnostic Horizons
 2:1-7, Microbiological Associates Quarterly Publication,
 Walkersville, MD); Voller, A. et al., 1978, J. Clin.
 Pathol. 31:507-520; Butler, J.E., 1981, Meth. Enzymol.
 73:482-523; Maggio, E. (ed.), 1980, Enzyme Immunoassay,
 15 CRC Press, Boca Raton, FL; Ishikawa, E. et al., (eds.),
 1981, Enzyme Immunoassay, Kigaku Shoin, Tokyo). The enzyme
 which is bound to the antibody will react with an
 appropriate substrate, preferably a chromogenic
 20 moiety which can be detected, for example, by
 spectrophotometric, fluorimetric or by visual means.
 Enzymes which can be used to detectably label the
 antibody include, but are not limited to, malate
 dehydrogenase, staphylococcal nuclease, delta-5-steroid
 25 isomerase, yeast alcohol dehydrogenase,
 alpha-glycerophosphate, dehydrogenase, triose phosphate
 isomerase, horseradish peroxidase, alkaline phosphatase,
 asparaginase, glucose oxidase, beta-galactosidase,
 ribonuclease, urease, catalase, glucose-6-phosphate
 30 dehydrogenase, glucoamylase and acetylcholinesterase. The
 detection can be accomplished by colorimetric methods
 which employ a chromogenic substrate for the enzyme.
 Detection may also be accomplished by visual comparison
 of the extent of enzymatic reaction of a substrate in
 35 comparison with similarly prepared standards.

Detection may also be accomplished using any of a
 variety of other immunoassays. For example, by

radioactively labeling the antibodies or antibody
5 fragments, it is possible to detect HBMYCNG gene peptides
through the use of a radioimmunoassay (RIA) (see, for
example, Weintraub, B., Principles of Radioimmunoassays,
Seventh Training Course on Radioligand Assay Techniques,
The Endocrine Society, March, 1986. The radioactive
10 isotope can be detected by such means as the use of a
gamma counter or a scintillation counter or by
autoradiography.

It is also possible to label the antibody with a
fluorescent compound. When the fluorescently labeled
15 antibody is exposed to light of the proper wave length,
its presence can then be detected due to fluorescence.
Among the most commonly used fluorescent labeling
compounds are fluorescein isothiocyanate, rhodamine,
phycoerythrin, phycocyanin, allophycocyanin,
20 o-phthaldehyde and fluorescamine.

The antibody can also be detectably labeled using
fluorescence emitting metals such as ^{152}Eu , or others of
the lanthanide series. These metals can be attached to
the antibody using such metal chelating groups as
25 diethylenetriaminepentacetic acid (DTPA) or
ethylenediaminetetraacetic acid (EDTA).

The antibody also can be detectably labeled by
coupling it to a chemiluminescent compound. The presence
of the chemiluminescent-tagged antibody is then
30 determined by detecting the presence of luminescence that
arises during the course of a chemical reaction. Examples
of particularly useful chemiluminescent labeling
compounds are luminol, isoluminol, theromatic acridinium
ester, imidazole, acridinium salt and oxalate ester.

35 Likewise, a bioluminescent compound may be used to
label the antibody of the present invention.
Bioluminescence is a type of chemiluminescence found in

biological systems in which a catalytic protein increases
5 the efficiency of the chemiluminescent reaction. The
presence of a bioluminescent protein is determined by
detecting the presence of luminescence. Important
bioluminescent compounds for purposes of labeling are
luciferin, luciferase and aequorin.

10

5.4.2. Screening Assays for Compounds That Modulate
HBMYCNG Activity

Screening assays can be used to identify compounds
that modulate HBMYCNG activity. These compounds can
15 include, but are not limited to, peptides, small organic
or inorganic molecules or macromolecules such as nucleic
acid molecules or proteins, and may be utilized, e.g., in
the control of ion and cation-related disorders, in the
modulation of cellular processes such as the release of
20 neurotransmitters or other cellular regulatory factors,
cell activation or regulation, cell death and changes in
cell membrane properties. These compounds may also be
useful, e.g., in elaborating the biological functions of
HBMYCNG gene products, modulating those biological
25 functions and for ameliorating symptoms of ion or
cation-related disorders.

The compositions of the invention include
pharmaceutical compositions comprising one or more of
these compounds. Such pharmaceutical compositions can be
30 formulated as discussed in Section 5.5, *infra*.

More specifically, these compounds can include
compounds that bind to HBMYCNG gene products, compounds
that bind to other proteins that interact with a HBMYCNG
gene product and/or interfere with the interaction of the
35 HBMYCNG gene product with other proteins, and compounds
that modulate the activity of the HBMYCNG gene, i.e.,

- modulate the level of HBMYCNG gene expression and/or
- 5 modulate the level of HBMYCNG gene product activity.

For example, assays may be utilized that identify compounds that bind to HBMYCNG gene regulatory sequences, e.g., promoter sequences (see e.g., Platt, K.A., 1994, J. Biol. Chem. 269:28558-28562), which compounds may

10 modulate the level of HBMYCNG gene expression. In addition, functional assays can be used to screen for compounds that modulate HBMYCNG gene product activity. In such assays, compounds are screened for agonistic or antagonistic activity with respect to a biological

15 activity or function of the HBMYCNG gene product, such as changes in the intracellular levels of an ion or cation, changes in regulatory factor release, or other activities or functions of the HBMYCNG polypeptides of the invention.

20 According to a preferred embodiment, a Ca^{2+} flux assay can be utilized to monitor calcium uptake in HBMYCNG-expressing host cells. The host cells are pre-loaded with a Ca^{2+} -sensitive fluorescently-labeled dye (e.g., Fluo-4, Fluo-3, Indo-1 or Fura-2), i.e., the

25 intracellular calcium is fluorescently labelled with the dye, and the effect of the compound, e.g., on the intracellular levels of the labeled-calcium determined and compared to the intracellular levels of control cells, e.g., lacking exposure to the compound of

30 interest. Compounds that have an agonistic, i.e., stimulatory, modulatory effect on HBMYCNG activity are those that, when contacted with the HBMYCNG-expressing cells, produce an increase in intracellular calcium relative to the control cells, whereas those compounds

35 having an antagonistic modulatory effect on HBMYCNG activity will be those that block the effects of agonists or cyclic nucleotides that increase channel activity. A

Ca²⁺ flux assay is exemplified in Example Section 6.1,

5 *infra*.

Functional assays for monitoring the effects of compounds on the levels or flux of other ions can be similarly performed; for example, the levels of potassium can be monitored using rubidium influx.

10 Screening assays may also be designed to identify compounds capable of binding to the HBMYCNG gene products of the invention. Such compounds may be useful, e.g., in modulating the activity of wild type and/or mutant HBMYCNG gene products, in elaborating the biological
15 function of the HBMYCNG gene product, and in screens for identifying compounds that disrupt normal HBMYCNG gene product interactions, or may in themselves disrupt such interactions.

The principle of such screening assays to identify
20 compounds that bind to the HBMYCNG gene product involves preparing a reaction mixture of the HBMYCNG gene product and the test compound under conditions and for a time sufficient to allow the two components to interact with, i.e., bind to, and thus form a complex, which can
25 represent a transient complex, which can be removed and/or detected in the reaction mixture. For example, one assay involves anchoring a HBMYCNG gene product or the test substance onto a solid phase and detecting HBMYCNG gene product/test compound complexes anchored on the
30 solid phase at the end of the reaction. In one embodiment of such a method, the HBMYCNG gene product may be anchored onto a solid surface, and the test compound, which is not anchored, may be labeled, either directly or indirectly.

35 The detection of complexes anchored on the solid surface can be accomplished in a number of ways. Where the previously non-immobilized component is pre-labeled,

the detection of label immobilized on the surface
5 indicates that complexes were formed. Where the
previously non-immobilized component is not pre-labeled,
an indirect label can be used to detect complexes
anchored on the surface; e.g., using a labeled antibody
specific for the previously non-immobilized component
10 (the antibody, in turn, may be directly labeled or
indirectly labeled with a labeled anti-Ig antibody).

Alternatively, a reaction can be conducted in a
liquid phase, the reaction products separated from
unreacted components, and complexes detected; e.g., using
15 an immobilized antibody specific for HBMYCNG gene product
or the test compound to anchor any complexes formed in
solution, and a labeled antibody specific for the other
component of the possible complex to detect anchored
complexes.

20 Compounds that modulate HBMYCNG gene product
activity can also include compounds that bind to proteins
that interact with the HBMYCNG gene product. These
modulatory compounds can be identified by first
identifying those proteins that interact with the HBMYCNG
25 gene product, e.g., by standard techniques known in the
art for detecting protein-protein interactions, such as
co-immunoprecipitation, crosslinking and co-purification
through gradients or chromatographic columns. Utilizing
procedures such as these allows for the isolation of
30 proteins that interact with HBMYCNG gene products or
polypeptides of the invention as described *supra*.

Once isolated, such a protein can be identified and
can, in turn, be used, in conjunction with standard
techniques, to identify additional proteins with which it
35 interacts. For example, at least a portion of the amino
acid sequence of the protein that interacts with the
HBMYCNG gene product can be ascertained using techniques

well known to those of skill in the art, such as via the
5 Edman degradation technique (see, e.g., Creighton, 1983,
"Proteins: Structures and Molecular Principles", W.H.
Freeman & Co., N.Y., pp.34-49). The amino acid sequence
thus obtained may be used as a guide for the generation
10 of oligonucleotide mixtures that can be used to screen
for gene sequences encoding such proteins. Screening may
be accomplished, for example, by standard hybridization
or PCR techniques. Techniques for the generation of
oligonucleotide mixtures and screening are well-known
(see, e.g., Ausubel, *supra.*, and PCR Protocols: A Guide
15 to Methods and Applications, 1990, Innis, M. et al., eds.
Academic Press, Inc., New York).

Additionally, methods may be employed that result in
the simultaneous identification of genes which encode
proteins interacting with HBMYCNG gene products or
20 polypeptides. These methods include, for example, probing
expression libraries with labeled HBMYCNG protein, using
HBMYCNG protein in a manner similar to the well known
technique of antibody probing of λ gt11 libraries. One
method that detects protein interactions *in vivo* is the
25 two-hybrid system. A version of this system is described
by Chien et al., 1991, Proc. Natl. Acad. Sci. USA,
88:9578-9582 and is commercially available from Clontech
(Palo Alto, CA).

In addition, compounds that disrupt HBMYCNG
30 interactions with its interacting or binding partners, as
determined immediately above, may be useful in regulating
the activity of the HBMYCNG gene product, including
mutant HBMYCNG gene products. Such compounds may include,
but are not limited to molecules such as peptides, and
35 the like, which may bind to the HBMYCNG gene product as
described above.

The basic principle of the assay systems used to
5 identify compounds that interfere with the interaction
between the HBMYCNG gene product and its interacting
partner or partners involves preparing a reaction mixture
containing the HBMYCNG gene product, and the interacting
10 partner under conditions and for a time sufficient to
allow the two to interact and bind, thus forming a
complex. In order to test a compound for inhibitory
activity, the reaction mixture is prepared in the
presence and absence of the test compound. The test
15 compound may be initially included in the reaction
mixture, or may be added at a time subsequent to the
addition of HBMYCNG gene product and its interacting
partner. Control reaction mixtures are incubated without
the test compound or with a placebo. The formation of any
20 complexes between the HBMYCNG gene product and the
interacting partner is then detected. The formation of a
complex in the control reaction, but not in the reaction
mixture containing the test compound, indicates that the
compound interferes with the interaction of the HBMYCNG
25 gene product and the interacting partner. Additionally,
complex formation within reaction mixtures containing the
test compound and a normal HBMYCNG gene product may also
be compared to complex formation within reaction mixtures
containing the test compound and a mutant HBMYCNG gene
30 product. This comparison may be important in those cases
wherein it is desirable to identify compounds that
disrupt interactions of mutant but not normal HBMYCNG
proteins.

The assay for compounds that interfere with the
interaction of HBMYCNG gene products and interacting
35 partners can be conducted in a heterogeneous or
homogeneous format. Heterogeneous assays involve
anchoring either the HBMYCNG gene product or the binding

partner onto a solid phase and detecting complexes
 5 anchored on the solid phase at the end of the reaction.
 In homogeneous assays, the entire reaction is carried out
 in a liquid phase. In either approach, the order of
 addition of reactants can be varied to obtain different
 information about the compounds being tested. For
 10 example, test compounds that interfere with the
 interaction between the HBMYCNG gene products and the
 interacting partners, e.g., by competition, can be
 identified by conducting the reaction in the presence of
 the test substance; i.e., by adding the test substance to
 15 the reaction mixture prior to or simultaneously with the
 HBMYCNG gene product and interacting partner.
 Alternatively, test compounds that disrupt preformed
 complexes, e.g., compounds with higher binding constants
 that displace one of the components from the complex, can
 20 be tested by adding the test compound to the reaction
 mixture after complexes have been formed. The various
 formats are described briefly below.

In a heterogeneous assay system, either the HBMYCNG
 gene product or the interacting partner, is anchored onto
 25 a solid surface, while the non-anchored species is
 labeled, either directly or indirectly. In practice,
 microtiter plates are conveniently utilized. The anchored
 species may be immobilized by non-covalent or covalent
 attachments. Non-covalent attachment may be accomplished
 30 simply by coating the solid surface with a solution of
 the HBMYCNG gene product or interacting partner and
 drying. Alternatively, an immobilized antibody specific
 for the species to be anchored may be used to anchor the
 species to the solid surface. The surfaces may be
 35 prepared in advance and stored.

In order to conduct the assay, the partner of the
 immobilized species is exposed to the coated surface with

or without the test compound. After the reaction is
5 complete, unreacted components are removed (e.g., by
washing) and any complexes formed will remain immobilized
on the solid surface. The detection of complexes anchored
on the solid surface can be accomplished in a number of
ways. Where the non-immobilized species is pre-labeled,
10 the detection of label immobilized on the surface
indicates that complexes were formed. Where the
non-immobilized species is not pre-labeled, an indirect
label can be used to detect complexes anchored on the
surface; e.g., using a labeled antibody specific for the
15 initially non-immobilized species (the antibody, in turn,
may be directly labeled or indirectly labeled with a
labeled anti-Ig antibody). Depending upon the order of
addition of reaction components, test compounds which
inhibit complex formation or which disrupt preformed
20 complexes can be detected.

Alternatively, the reaction can be conducted in a
liquid phase in the presence or absence of the test
compound, the reaction products separated from unreacted
components, and complexes detected; e.g., using an
25 immobilized antibody specific for one of the interacting
components to anchor any complexes formed in solution,
and a labeled antibody specific for the other partner to
detect anchored complexes. Again, depending upon the
order of addition of reactants to the liquid phase, test
30 compounds that inhibit complex formation or that disrupt
preformed complexes can be identified.

In an alternate embodiment, a preformed complex of
the HBMYCNG gene protein and the interacting partner is
prepared in which either the HBMYCNG gene product or its
35 interacting partners is labeled, but the signal generated
by the label is quenched due to complex formation (see,
e.g., U.S. Patent No. 4,109,496 by Rubenstein which

utilizes this approach for immunoassays). The addition of
5 a test substance that competes with and displaces one of
the species from the preformed complex will result in the
generation of a signal above background. In this way,
test substances that disrupt HBMYCNG gene
protein/interacting partner interaction can be
10 identified.

In another embodiment of the invention, these same
techniques can be employed using peptide fragments that
correspond to the binding domains of the HBMYCNG protein
and/or the interacting partner, in place of one or both
15 of the full length proteins. Any number of methods
routinely practiced in the art can be used to identify
and isolate the binding sites. These methods include, but
are not limited to, mutagenesis of the gene encoding one
of the proteins and screening for disruption of binding
20 in a co-immunoprecipitation assay. Compensating mutations
in the gene encoding the second species in the complex
can then be selected. Sequence analysis of the genes
encoding the respective proteins will reveal the
mutations that correspond to the region of the protein
25 involved in interacting, e.g., binding. Alternatively,
one protein can be anchored to a solid surface using
methods described in this Section above, and allowed to
interact with, e.g., bind, to its labeled interacting
partner, which has been treated with a proteolytic
30 enzyme, such as trypsin. After washing, a short, labeled
peptide comprising the interacting, e.g., binding, domain
may remain associated with the solid material, which can
be isolated and identified by amino acid sequencing.
Also, once the gene coding for the intracellular binding
35 partner is obtained, short gene segments can be
engineered to express peptide fragments of the protein,

which can then be tested for binding activity and
5 purified or synthesized.

The human HBMYCNG polypeptides and/or peptides of
the present invention, or immunogenic fragments or
oligopeptides thereof, can be used for screening
therapeutic drugs or compounds in a variety of drug
10 screening techniques. The fragment employed in such a
screening assay may be free in solution, affixed to a
solid support, borne on a cell surface, or located
intracellularly. The reduction or abolition of activity
of the formation of binding complexes between the ion
15 channel protein and the agent being tested can be
measured. Thus, the present invention provides a method
for screening or assessing a plurality of compounds for
their specific binding affinity with a HBMYCNG
polypeptide, or a bindable peptide fragment, of this
20 invention, comprising providing a plurality of compounds,
combining the HBMYCNG polypeptide, or a bindable peptide
fragment, with each of a plurality of compounds for a
time sufficient to allow binding under suitable
conditions and detecting binding of the HBMYCNG
25 polypeptide or peptide to each of the plurality of test
compounds, thereby identifying the compounds that
specifically bind to the HBMYCNG polypeptide or peptide.

Methods of identifying compounds that modulate the
activity of the novel human HBMYCNG polypeptides and/or
30 peptides are provided by the present invention and
comprise combining a potential or candidate compound or
drug modulator of ion channel biological activity with an
HBMYCNG polypeptide or peptide, for example, the HBMYCNG
amino acid sequence as set forth in SEQ ID NOS:2, and
35 measuring an effect of the candidate compound or drug
modulator on the biological activity of the HBMYCNG
polypeptide or peptide. Such measurable effects include,
for example, physical binding interaction; the ability to

5 cleave a suitable ion channel substrate; effects on native and cloned HBMYCNG-expressing cell line; and effects of modulators or other ion channel-mediated physiological measures.

Another method of identifying compounds that modulate the biological activity of the novel HBMYCNG polypeptides of the present invention comprises combining
10 a potential or candidate compound or drug modulator of a ion channel biological activity with a host cell that expresses the HBMYCNG polypeptide and measuring an effect of the candidate compound or drug modulator on the
15 biological activity of the HBMYCNG polypeptide. The host cell can also be capable of being induced to express the HBMYCNG polypeptide, e.g., via inducible expression. Physiological effects of a given modulator candidate on the HBMYCNG polypeptide can also be measured. Thus,
20 cellular assays for particular ion channel modulators may be either direct measurement or quantification of the physical biological activity of the HBMYCNG polypeptide, or they may be measurement or quantification of a physiological effect. Such methods preferably employ a HBMYCNG polypeptide as described herein, or an
25 overexpressed recombinant HBMYCNG polypeptide in suitable host cells containing an expression vector as described herein, wherein the HBMYCNG polypeptide is expressed, overexpressed, or undergoes upregulated expression.

Another aspect of the present invention embraces a
30 method of screening for a compound that is capable of modulating the biological activity of a HBMYCNG polypeptide, comprising providing a host cell containing an expression vector harboring a nucleic acid sequence encoding a HBMYCNG polypeptide, or a functional peptide
35 or portion thereof (e.g., SEQ ID NOS:2); determining the biological activity of the expressed HBMYCNG polypeptide in the absence of a modulator compound; contacting the

cell with the modulator compound and determining the
5 biological activity of the expressed HBMYCNG polypeptide
in the presence of the modulator compound. In such a
method, a difference between the activity of the HBMYCNG
polypeptide in the presence of the modulator compound and
in the absence of the modulator compound indicates a
10 modulating effect of the compound.

Essentially any chemical compound can be employed as
a potential modulator or ligand in the assays according
to the present invention. Compounds tested as ion channel
modulators can be any small chemical compound, or
15 biological entity (e.g., protein, sugar, nucleic acid,
lipid). Test compounds will typically be small chemical
molecules and peptides. Generally, the compounds used as
potential modulators can be dissolved in aqueous or
organic (e.g., DMSO-based) solutions. The assays are
20 designed to screen large chemical libraries by automating
the assay steps and providing compounds from any
convenient source. Assays are typically run in parallel,
for example, in microtiter formats on microtiter plates
in robotic assays. There are many suppliers of chemical
25 compounds, including Sigma (St. Louis, MO), Aldrich (St.
Louis, MO), Sigma-Aldrich (St. Louis, MO), Fluka Chemika-
Biochemica Analytika (Buchs, Switzerland), for example.
Also, compounds may be synthesized by methods known in
the art.

High throughput screening methodologies are
30 particularly envisioned for the detection of modulators
of the novel HBMYCNG polynucleotides and polypeptides
described herein. Such high throughput screening methods
typically involve providing a combinatorial chemical or
peptide library containing a large number of potential
35 therapeutic compounds (e.g., ligand or modulator
compounds). Such combinatorial chemical libraries or
ligand libraries are then screened in one or more assays

to identify those library members (e.g., particular
5 chemical species or subclasses) that display a desired
characteristic activity. The compounds so identified can
serve as conventional lead compounds, or can themselves
be used as potential or actual therapeutics.

A combinatorial chemical library is a collection of
10 diverse chemical compounds generated either by chemical
synthesis or biological synthesis, by combining a number
of chemical building blocks (i.e., reagents such as amino
acids). As an example, a linear combinatorial library,
e.g., a polypeptide or peptide library, is formed by
15 combining a set of chemical building blocks in every
possible way for a given compound length (i.e., the
number of amino acids in a polypeptide or peptide
compound). Millions of chemical compounds can be
synthesized through such combinatorial mixing of chemical
20 building blocks.

The preparation and screening of combinatorial
chemical libraries is well known to those having skill in
the pertinent art. Combinatorial libraries include,
without limitation, peptide libraries (e.g. U.S. Patent
25 No. 5,010,175; Furka, 1991, *Int. J. Pept. Prot. Res.*,
37:487-493; and Houghton et al., 1991, *Nature*, 354:84-
88). Other chemistries for generating chemical diversity
libraries can also be used. Nonlimiting examples of
chemical diversity library chemistries include, peptoids
30 (PCT Publication No. WO 91/019735), encoded peptides (PCT
Publication No. WO 93/20242), random bio-oligomers (PCT
Publication No. WO 92/00091), benzodiazepines (U.S.
Patent No. 5,288,514), diversomers such as hydantoins,
benzodiazepines and dipeptides (Hobbs et al., 1993, *Proc.*
35 *Natl. Acad. Sci. USA*, 90:6909-6913), vinylogous
polypeptides (Hagihara et al., 1992, *J. Amer. Chem. Soc.*,
114:6568), nonpeptidal peptidomimetics with glucose
scaffolding (Hirschmann et al., 1992, *J. Amer. Chem.*

- Soc., 114:9217-9218), analogous organic synthesis of
- 5 small compound libraries (Chen et al., 1994, *J. Amer. Chem. Soc.*, 116:2661), oligocarbamates (Cho et al., 1993, *Science*, 261:1303), and/or peptidyl phosphonates (Campbell et al., 1994, *J. Org. Chem.*, 59:658), nucleic acid libraries (see Ausubel, Berger and Sambrook, all
- 10 supra), peptide nucleic acid libraries (U.S. Patent No. 5,539,083), antibody libraries (e.g., Vaughn et al., 1996, *Nature Biotechnology*, 14(3):309-314) and PCT/US96/10287), carbohydrate libraries (e.g., Liang et al., 1996, *Science*, 274-1520-1522) and U.S. Patent No.
- 15 5,593,853), small organic molecule libraries (e.g., benzodiazepines, Baum C&EN, Jan. 18, 1993, page 33; and U.S. Patent No. 5,288,514; isoprenoids, U.S. Patent No. 5,569,588; thiazolidinones and metathiazanones, U.S. Patent No. 5,549,974; pyrrolidines, U.S. Patent Nos.
- 20 5,525,735 and 5,519,134; morpholino compounds, U.S. Patent No. 5,506,337; and the like).

- Devices for the preparation of combinatorial libraries are commercially available (e.g., 357 MPS, 390 MPS, Advanced Chem Tech, Louisville KY; Symphony, Rainin,
- 25 Woburn, MA; 433A Applied Biosystems, Foster City, CA; 9050 Plus, Millipore, Bedford, MA). In addition, a large number of combinatorial libraries are commercially available (e.g., ComGenex, Princeton, NJ; Asinex, Moscow, Russia; Tripos, Inc., St. Louis, MO; ChemStar, Ltd.,
- 30 Moscow, Russia; 3D Pharmaceuticals, Exton, PA; Martek Biosciences, Columbia, MD, and the like).

- In one embodiment, the invention provides solid phase based *in vitro* assays in a high throughput format, where the cell or tissue expressing an ion channel is
- 35 attached to a solid phase substrate. In such high throughput assays, it is possible to screen up to several thousand different modulators or ligands in a single day. In particular, each well of a microtiter plate can be

used to perform a separate assay against a selected
5 potential modulator, or, if concentration or incubation
time effects are to be observed, every 5-10 wells can
test a single modulator. Thus, a single standard
microtiter plate can assay about 96 modulators. If 1536
well plates are used, then a single plate can easily
10 assay from about 100 to about 1500 different compounds.
It is possible to assay several different plates per day;
thus, for example, assay screens for up to about 6,000-
20,000 different compounds are possible using the
described integrated systems.

15 In another of its aspects, the present invention
encompasses screening and small molecule (e.g., drug)
detection assays which involve the detection or
identification of small molecules that can bind to a
given protein, i.e., a HBMYCNG polypeptide or peptide.
20 Particularly preferred are assays suitable for high
throughput screening methodologies.

In such binding-based detection, identification, or
screening assays, a functional assay is not typically
required. All that is needed is a target protein,
preferably substantially purified, and a library or panel
25 of compounds (e.g., ligands, drugs, small molecules) or
biological entities to be screened or assayed for binding
to the protein target. Preferably, most small molecules
that bind to the target protein will modulate activity in
some manner, due to preferential, higher affinity binding
30 to functional areas or sites on the protein.

An example of such an assay is the fluorescence
based thermal shift assay (3-Dimensional Pharmaceuticals,
Inc., 3DP, Exton, PA) as described in U.S. Patent Nos.
6,020,141 and 6,036,920 to Pantoliano et al.; see also,
35 J. Zimmerman, 2000, *Gen. Eng. News*, 20(8)). The assay
allows the detection of small molecules (e.g., drugs,
ligands) that bind to expressed, and preferably purified,

ion channel polypeptide based on affinity of binding
5 determinations by analyzing thermal unfolding curves of
protein-drug or ligand complexes. The drugs or binding
molecules determined by this technique can be further
assayed, if desired, by methods, such as those described
herein, to determine if the molecules affect or modulate
10 function or activity of the target protein.

To purify a HBMYCNG polypeptide or peptide to
measure a biological binding or ligand binding activity,
the source may be a whole cell lysate that can be
prepared by successive freeze-thaw cycles (e.g., one to
15 three) in the presence of standard protease inhibitors.
The HBMYCNG polypeptide may be partially or completely
purified by standard protein purification methods, e.g.,
affinity chromatography using specific antibody described
infra, or by ligands specific for an epitope tag
20 engineered into the recombinant HBMYCNG polypeptide
molecule, also as described herein. Binding activity can
then be measured as described.

Compounds which are identified according to the
methods provided herein, and which modulate or regulate
25 the biological activity or physiology of the HBMYCNG
polypeptides according to the present invention are a
preferred embodiment of this invention. It is
contemplated that such modulatory compounds may be
employed in treatment and therapeutic methods for
treating a condition that is mediated by the novel
30 HBMYCNG polypeptides by administering to an individual in
need of such treatment a therapeutically effective amount
of the compound identified by the methods described
herein.

In addition, the present invention provides methods
35 for treating an individual in need of such treatment for
a disease, disorder, or condition that is mediated by the
HBMYCNG polypeptides of the invention, comprising

administering to the individual a therapeutically
 5 effective amount of the HBMYCNG-modulating compound
 identified by a method provided herein.

5.4.3. Methods and Compositions for the Treatment of Ion Channel-Related Disorders

10 The present invention also relates to methods and
 compositions for the treatment or modulation of any
 disorder or cellular process that is mediated or
 regulated by HBMYCNG gene product expression or function,
 e.g., HBMYCNG-mediated cell activation, signal
 15 transduction, cellular regulatory factor release, etc.
 Further, HBMYCNG effector functions can be modulated via
 such methods and compositions.

The methods of the invention include methods that
 modulate HBMYCNG gene and gene product activity. In
 20 certain instances, the treatment will require an
 increase, upregulation or activation of HBMYCNG activity,
 while in other instances, the treatment will require a
 decrease, downregulation or suppression of HBMYCNG
 activity. "Increase" and "decrease" refer to the
 25 differential level of HBMYCNG activity relative to
 HBMYCNG activity in the cell type of interest in the
 absence of modulatory treatment. Methods for the decrease
 of HBMYCNG activity are discussed in Section 5.4.3.1,
infra. Methods for the increase of HBMYCNG activity are
 30 discussed in Section 5.4.3.2, *infra*. Methods which can
 either increase or decrease HBMYCNG activity depending on
 the particular manner in which the method is practiced
 are discussed in Section 5.4.3.3, *infra*.

35 5.4.3.1. Methods for Decreasing HBMYCNG Activity

Successful treatment of ion channel/ionic
 homeostasis disorders, e.g., CNS disorders, cardiac

disorders or hypercalcemia, can be brought about by
5 methods which serve to decrease HBMYCNG activity.
Activity can be decreased by, e.g., directly decreasing
HBMYCNG gene product activity and/or by decreasing the
level of HBMYCNG gene expression.

For example, compounds such as those identified
10 through assays described in Section 5.4.2., *supra*, that
decrease HBMYCNG gene product activity can be used in
accordance with the invention to ameliorate symptoms
associated with ion channel/ionic homeostasis disorders.
As discussed *supra*, such molecules can include, but are
15 not limited to peptides, including soluble peptides, and
small organic or inorganic molecules, and can be referred
to as HBMYCNG antagonists. Techniques for the
determination of effective doses and administration of
such compounds are described in Section 5.5., *infra*.

20 In addition, antisense and ribozyme molecules that
inhibit HBMYCNG gene expression can also be used to
reduce the level of HBMYCNG gene expression, thus
effectively reducing the level of HBMYCNG gene product
present, thereby decreasing the level of HBMYCNG
25 activity. Still further, triple helix molecules can be
utilized in reducing the level of HBMYCNG gene
expression. Such molecules can be designed to reduce or
inhibit either wild type, or if appropriate, mutant
target gene activity. Techniques for the production and
30 use of such molecules are well known to those of skill in
the art.

Antisense approaches involve the design of
oligonucleotides (either DNA or RNA) that are
complementary to HBMYCNG gene mRNA. The antisense
35 oligonucleotides will bind to the complementary HBMYCNG
gene mRNA transcripts and prevent translation. Absolute
complementarity, although preferred, is not required. A

sequence "complementary" to a portion of an RNA, as
5 referred to herein, means a sequence having sufficient
complementarity to be able to hybridize with the RNA,
forming a stable duplex; in the case of double-stranded
antisense nucleic acids, a single strand of the duplex
DNA may thus be tested, or triplex formation may be
10 assayed. The ability to hybridize will depend on both the
degree of complementarity and the length of the antisense
nucleic acid. Generally, the longer the hybridizing
nucleic acid, the more base mismatches with an RNA it may
contain and still form a stable duplex (or triplex, as
15 the case may be). One skilled in the art can ascertain a
tolerable degree of mismatch by use of standard
procedures to determine the melting point of the
hybridized complex.

Oligonucleotides that are complementary to the 5'
20 end of the message, e.g., the 5' untranslated sequence up
to and including the AUG initiation codon, should work
most efficiently at inhibiting translation. However,
sequences complementary to the 3' untranslated sequences
of mRNAs have recently been shown to be effective at
25 inhibiting translation of mRNAs as well. See generally,
Wagner, R., 1994, Nature 372:333-335. Thus,
oligonucleotides complementary to either the 5'- or 3'-
non-translated, non-coding regions of the HBMYCNG gene,
as depicted in FIG. 1 could be used in an antisense
30 approach to inhibit translation of endogenous HBMYCNG
gene mRNA.

Oligonucleotides complementary to the 5'
untranslated region of the mRNA should include the
complement of the AUG start codon. Antisense
35 oligonucleotides complementary to mRNA coding regions are
less efficient inhibitors of translation but could be
used in accordance with the invention. Whether designed

to hybridize to the 5'-, 3'- or coding region of target
 5 or pathway gene mRNA, antisense nucleic acids should be
 at least six nucleotides in length, and are preferably
 oligonucleotides ranging from 6 to about 50 nucleotides
 in length. In specific aspects, the oligonucleotide is at
 least 10 nucleotides, at least 17 nucleotides, at least
 10 25 nucleotides or at least 50 nucleotides.

Regardless of the choice of target sequence, it is
 preferred that *in vitro* studies are first performed to
 quantitate the ability of the antisense oligonucleotide
 to inhibit gene expression. It is preferred that these
 15 studies utilize controls that distinguish between
 antisense gene inhibition and non-specific biological
 effects of oligonucleotides. It is also preferred that
 these studies compare levels of the target RNA or protein
 with that of an internal control RNA or protein.
 20 Additionally, results obtained using the antisense
 oligonucleotide are preferably compared with those
 obtained using a control oligonucleotide. It is preferred
 that the control oligonucleotide is of approximately the
 same length as the antisense oligonucleotide and that the
 25 nucleotide sequence of the control oligonucleotide
 differs from the antisense sequence no more than is
 necessary to prevent specific hybridization to the target
 sequence.

The oligonucleotides can be DNA or RNA or chimeric
 30 mixtures or derivatives or modified versions thereof,
 single-stranded or double-stranded. The oligonucleotide
 can be modified at the base moiety, sugar moiety, or
 phosphate backbone, for example, to improve stability of
 the molecule, hybridization, etc.

35 The oligonucleotide may also include other appended
 groups such as peptides (e.g., for targeting host cell
 receptors *in vivo*), or agents facilitating transport

- across the cell membrane (see, e.g., Letsinger et al.,
 5 1989, Proc. Natl. Acad. Sci. U.S.A. 86:6553-6556;
 Lemaitre et al., 1987, Proc. Natl. Acad. Sci. 84:648-652;
 PCT Application No.
 WO 88/09810) or the blood-brain barrier (see, e.g., PCT
 Application No. WO 89/10134), or hybridization-triggered
 10 cleavage agents (see, e.g., Krol et al., 1988,
 BioTechniques 6:958-976) or intercalating agents (see,
 e.g., Zon, 1988, Pharm. Res. 5:539-549). For example, the
 oligonucleotide may be conjugated to another molecule,
 e.g., a peptide, hybridization triggered cross-linking
 15 agent, transport agent, hybridization-triggered cleavage
 agent, etc.

- Oligonucleotides of the invention may be synthesized
 by standard methods known in the art, e.g., by use of an
 automated DNA synthesizer (such as are commercially
 20 available from Biosearch, Applied Biosystems, etc.). As
 examples, phosphorothioate oligonucleotides may be
 synthesized by the method of Stein et al. (1988, Nucl.
 Acids Res. 16:3209) and methylphosphonate
 oligonucleotides can be prepared by use of controlled
 25 pore glass polymer supports (Sarin et al., 1988, Proc.
 Natl. Acad. Sci. U.S.A. 85:7448-7451), etc.

- The antisense molecules should be delivered to cells
 which express the HBMYCNG gene *in vivo*. A number of
 methods have been developed for delivering antisense DNA
 30 or RNA to cells; e.g., antisense molecules can be
 injected directly into the tissue site or modified
 antisense molecules designed to target the desired cells
 (e.g., antisense linked to peptides or antibodies that
 specifically bind receptors or antigens expressed on the
 35 target cell surface) can be administered systemically.

However, it is often difficult to achieve
 intracellular concentrations of the antisense sufficient

to suppress translation of endogenous mRNAs. Thus, a
5 preferred approach utilizes a recombinant DNA construct
in which the antisense oligonucleotide is placed under
the control of a strong pol III or pol II promoter. The
use of such a construct to transfect target cells in the
patient will result in the transcription of sufficient
10 amounts of single stranded RNAs that will form
complementary base pairs with the endogenous HBMYCNG gene
transcripts and thereby prevent translation of the
HBMYCNG gene mRNA. For example, a vector can be
introduced *in vivo* such that it is taken up by a cell and
15 directs the transcription of an antisense RNA.

Ribozymes are enzymatic RNA molecules capable of
catalyzing the specific cleavage of RNA (For a review,
see, e.g., Rossi, J., 1994, Current Biology 4:469-471).
The mechanism of ribozyme action involves
20 sequence-specific hybridization of the ribozyme molecule
to complementary target RNA, followed by a
endonucleolytic cleavage. The composition of ribozyme
molecules must include one or more sequences
complementary to the target gene mRNA, and must include
25 the well known catalytic sequence responsible for mRNA
cleavage. For this sequence, see United States Patent No.
5,093,246, which is incorporated by reference herein in
its entirety. As such, within the scope of the invention
are engineered hammerhead motif ribozyme molecules that
30 specifically and efficiently catalyze endonucleolytic
cleavage of RNA sequences encoding target gene proteins.

Ribozyme molecules designed to catalytically cleave
HBMYCNG gene mRNA transcripts can also be used to prevent
translation of HBMYCNG gene mRNA and expression of target
35 or pathway genes. (See, e.g., PCT Application No. WO
90/11364; Sarver et al., 1990, Science 247:1222-1225).

The ribozymes of the present invention also include
 5 RNA endoribonucleases (hereinafter referred to as
 "Cech-type ribozymes") such as the one which occurs
 naturally in *Tetrahymena Thermophila* (known as the IVS,
 or L-19 IVS RNA) and which has been extensively described
 by Thomas Cech and collaborators (Zaug, et al., 1984,
 10 Science, 224:574-578; Zaug and Cech, 1986, Science,
 231:470-475; Zaug, et al., 1986, Nature, 324:429-433; PCT
 Patent Application No. WO 88/04300; Been and Cech, 1986,
 Cell, 47:207-216). The Cech-type ribozymes have an eight
 15 base pair active site which hybridizes to a target RNA
 sequence, after which cleavage of the target RNA takes
 place. The invention encompasses those Cech-type
 ribozymes which target eight base-pair active site
 sequences that are present in an HBMYCNG gene.

As in the antisense approach, the ribozymes can be
 20 composed of modified oligonucleotides (e.g. for improved
 stability, targeting, etc.) and should be delivered to
 cells which express the HBMYCNG gene *in vivo*. A preferred
 method of delivery involves using a DNA construct
 "encoding" the ribozyme under the control of a strong
 25 constitutive pol III or pol II promoter, so that
 transfected cells will produce sufficient quantities of
 the ribozyme to destroy endogenous HBMYCNG gene messages
 and inhibit translation. Because ribozymes, unlike
 antisense molecules, are catalytic, a lower intracellular
 30 concentration is required for efficiency.

Endogenous HBMYCNG gene expression can also be
 reduced by inactivating or "knocking out" the target
 and/or pathway gene or its promoter using targeted
 homologous recombination (see, e.g., Smithies et al.,
 35 1985, Nature 317:230-234; Thomas & Capecchi, 1987, Cell
 51:503-512; Thompson et al., 1989 Cell 5:313-321). For
 example, a mutant, non-functional HBMYCNG gene (or a

- completely unrelated DNA sequence) flanked by DNA
- 5 homologous to the endogenous HBMYCNG gene (either the coding regions or regulatory regions of the HBMYCNG gene) can be used, with or without a selectable marker and/or a negative selectable marker, to transfect cells that express the HBMYCNG gene *in vivo*. Insertion of the DNA
 - 10 construct, via targeted homologous recombination, results in inactivation of the HBMYCNG gene. Such techniques can also be utilized to generate ion/cation disorder animal models. It should be noted that this approach can be adapted for use in humans provided the recombinant DNA
 - 15 constructs are directly administered or targeted to the required site *in vivo* using appropriate viral vectors, e.g., herpes virus vectors.

- Alternatively, endogenous HBMYCNG gene expression can be reduced by targeting deoxyribonucleotide sequences
- 20 complementary to the regulatory region of the HBMYCNG gene (i.e., the HBMYCNG gene promoter and/or enhancers) to form triple helical structures that prevent transcription of the HBMYCNG gene in target cells in the body (see generally, Helene, C., 1991, Anticancer Drug
 - 25 Des. 6(6):569-84; Helene, C., et al., 1992, Ann. N.Y. Acad. Sci. 660:27-36; and Maher, L.J., 1992, Bioassays 14(12):807-15).

- Nucleic acid molecules to be used in triple helix formation for the inhibition of transcription should be
- 30 single stranded and composed of deoxynucleotides. The base composition of these oligonucleotides should be designed to promote triple helix formation via Hoogsteen base pairing rules, which generally require sizeable stretches of either purines or pyrimidines to be present
 - 35 on one strand of the duplex. Nucleotide sequences may be pyrimidine-based, which will result in TAT and CGC+ triplets across the three associated strands of the

5 resulting triple helix. The pyrimidine-rich molecules provide base complementarity to a purine-rich region of a single strand of the duplex in a parallel orientation to that strand. In addition, nucleic acid molecules may be chosen that are purine-rich, for example, containing a stretch of G residues. These molecules will form a triple
10 helix with a DNA duplex that is rich in GC pairs, in which the majority of the purine residues are located on a single strand of the targeted duplex, resulting in GGC triplets across the three strands of the triplex.

Alternatively, the potential sequences that can be
15 targeted for triple helix formation may be increased by creating a "switchback" nucleic acid molecule. Switchback molecules are synthesized in an alternating 5'-3', 3'-5' manner, such that they base pair with first one strand of a duplex and then the other, eliminating the necessity
20 for a sizeable stretch of either purines or pyrimidines to be present on one strand of the duplex.

In instances wherein the antisense, ribozyme, and/or triple helix molecules described herein are utilized to inhibit mutant HBMYCNG gene expression, it is possible
25 that the technique may so efficiently reduce or inhibit the transcription (triple helix) and/or translation (antisense, ribozyme) of mRNA produced by normal target gene alleles that the concentration of normal target gene product present may be lower than is necessary for a
30 normal phenotype. In such cases, to ensure that substantially normal levels of HBMYCNG gene activity are maintained, nucleic acid molecules that encode and express HBMYCNG gene polypeptides exhibiting normal target gene activity can be introduced into cells via
35 gene therapy methods that do not contain sequences susceptible to whatever antisense, ribozyme, or triple helix treatments are being utilized. In instances where

the target gene encodes an extracellular protein, it can
 5 be preferable to coadminister normal target gene protein
 in order to maintain the requisite level of target gene
 activity.

Antisense RNA and DNA, ribozyme, and triple helix
 molecules of the invention can be prepared by any method
 10 known in the art, e.g., methods for chemically
 synthesizing oligodeoxyribonucleotides and
 oligoribonucleotides well known in the art such as solid
 phase phosphoramidite chemical synthesis. Alternatively,
 RNA molecules can be generated by *in vitro* and *in vivo*
 15 transcription of DNA sequences encoding the antisense RNA
 molecule. Such DNA sequences can be incorporated into a
 wide variety of vectors which incorporate suitable RNA
 polymerase promoters such as the T7 or SP6 polymerase
 promoters. Alternatively, antisense cDNA constructs that
 20 synthesize antisense RNA constitutively or inducibly,
 depending on the promoter used, can be introduced stably
 into cell lines.

In addition, well-known modifications to DNA
 molecules can be introduced into the HBMYCNG nucleic acid
 25 molecules of the invention as a means of increasing
 intracellular stability and half-life. Possible
 modifications include, but are not limited to, the
 addition of flanking sequences of ribo- or deoxy-
 nucleotides to the 5' and/or 3' ends of the molecule or
 30 the use of phosphorothioate or 2' O-methyl rather than
 phosphodiesterase linkages within the
 oligodeoxyribonucleotide backbone.

5.4.3.2. Methods for Increasing HBMYCNG Activity

35 Successful treatment of ion/cation disorders can
 also be brought about by techniques which serve to
 increase the level of HBMYCNG activity. Activity can be

increased by, for example, directly increasing HBMYCNG
5 gene product activity and/or by increasing the level of
HBMYCNG gene expression.

For example, compounds such as those identified
through the assays described in Section 5.4.2., *supra*,
that increase HBMYCNG activity can be used to treat
10 ion/cation-related disorders. Such molecules can include,
but are not limited to peptides, including soluble
peptides, and small organic or inorganic molecules, and
can be referred to as HBMYCNG agonists.

For example, a compound can, at a level sufficient
15 to treat ion/cation-related disorders and symptoms, be
administered to a patient exhibiting such symptoms. One
of skill in the art will readily know how to determine
the concentration of effective, non-toxic doses of the
compound, utilizing techniques such as those described
20 *infra*.

Alternatively, in instances wherein the compound to
be administered is a peptide compound, DNA sequences
encoding the peptide compound can be directly
administered to a patient exhibiting an
25 ion/cation-related disorder or symptoms, at a
concentration sufficient to produce a level of peptide
compound sufficient to ameliorate the symptoms of the
disorder. Any of the techniques discussed *infra*, which
achieve intracellular administration of compounds, such
30 as, for example, liposome administration, can be utilized
for the administration of such DNA molecules. In the case
of peptide compounds which act extracellularly, the DNA
molecules encoding such peptides can be taken up and
expressed by any cell type, so long as a sufficient
35 circulating concentration of peptide results for the
elicitation of a reduction in the ion/cation disorder
symptoms.

In cases where the ion/cation disorder can be
5 localized to a particular portion or region of the body,
the DNA molecules encoding such modulatory peptides may
be administered as part of a delivery complex. Such a
delivery complex can comprise an appropriate nucleic acid
molecule and a targeting means. Such targeting means can
10 comprise, for example, sterols lipids, viruses or target
cell specific binding agents. Viral vectors can include,
but are not limited to adenovirus, adeno-associated
virus, and retrovirus vectors, in addition to other
particles that introduce DNA into cells, such as
15 liposomes.

Further, in instances wherein the ion/cation-related
disorder involves an aberrant HBMYCNG gene, patients can
be treated by gene replacement therapy. One or more
copies of a normal HBMYCNG gene or a portion of the gene
20 that directs the production of a normal HBMYCNG gene
protein with HBMYCNG gene function, can be inserted into
cells, via, for example a delivery complex as described
supra.

Such gene replacement techniques can be accomplished
25 either *in vivo* or *in vitro*. Techniques which select for
expression within the cell type of interest are
preferred. For *in vivo* applications, such techniques can,
for example, include appropriate local administration of
HBMYCNG gene sequences.

30 Additional methods which may be utilized to increase
the overall level of HBMYCNG activity include the
introduction of appropriate HBMYCNG gene-expressing
cells, preferably autologous cells, into a patient at
positions and in numbers which are sufficient to
35 ameliorate the symptoms of the ion/cation-related
disorder. Such cells may be either recombinant or
non-recombinant. Among the cells which can be

administered to increase the overall level of HBMYCNG gene expression in a patient are normal cells, which express the HBMYCNG gene. The cells can be administered at the anatomical site of expression, or as part of a tissue graft located at a different site in the body. Such cell-based gene therapy techniques are well known to those skilled in the art (see, e.g., Anderson, et al., United States Patent No. 5,399,349; Mulligan and Wilson, United States Patent No. 5,460,959).

HBMYCNG gene sequences can also be introduced into autologous cells *in vitro*. These cells expressing the HBMYCNG gene sequence can then be reintroduced, preferably by intravenous administration, into the patient until the disorder is treated and symptoms of the disorder are ameliorated.

20 5.4.3.3. Additional Modulatory Techniques

The present invention also includes modulatory techniques which, depending on the specific application for which they are utilized, can yield either an increase or a decrease in HBMYCNG activity levels leading to the amelioration of ion/cation-related disorders such as those described above.

Antibodies exhibiting modulatory capability can be utilized according to the methods of this invention to treat the ion/cation-related disorders. Depending on the specific antibody, the modulatory effect can be an increase or decrease in HBMYCNG activity. Such antibodies can be generated using standard techniques described in Section 5.3, *supra*, against full length wild type or mutant HBMYCNG proteins, or against peptides corresponding to portions of the proteins, as well as against extracellular domains of the HBMYCNG polypeptide or HBMYCNG epitopes within the water soluble fusion

protein mimic of the HMBYCNG disclosed above. The
 5 antibodies include but are not limited to polyclonal,
 monoclonal, Fab fragments, single chain antibodies,
 chimeric antibodies, etc.

Lipofectin or liposomes can be used to deliver the
 antibody or a fragment of the Fab region which binds to
 10 the HMBYCNG gene product epitope to cells expressing the
 gene product. Where fragments of the antibody are used,
 the smallest inhibitory fragment which binds to the
 HMBYCNG protein's binding domain is preferred. For
 example, peptides having an amino acid sequence
 15 corresponding to the domain of the variable region of the
 antibody that binds to the HMBYCNG protein can be used.
 Such peptides can be synthesized chemically or produced
 via recombinant DNA technology using methods well known
 in the art (e.g., see Creighton, 1983, *supra* and Sambrook
 20 et al., 1989, *supra*). Alternatively, single chain
 antibodies, such as neutralizing antibodies, which bind
 to intracellular epitopes can also be administered. Such
 single chain antibodies can be administered, for example,
 by expressing nucleotide sequences encoding single-chain
 25 antibodies within the target cell population by
 utilizing, for example, techniques such as those
 described in Marasco et al., 1993, Proc. Natl. Acad. Sci.
 USA 90:7889-7893.

30 5.5. Pharmaceutical Preparations And Methods of Administration

The compounds, e.g., nucleic acid sequences,
 polypeptides, peptides, and recombinant cells, described
supra can be administered to a patient at therapeutically
 35 effective doses to treat or ameliorate ion/cation-related
 disorders. A therapeutically effective dose refers to
 that amount of a compound or cell population sufficient

to result in amelioration of the disorder symptoms, or
5 alternatively, to that amount of a nucleic acid sequence
sufficient to express a concentration of HBMYCNG gene
product which results in the amelioration of the disorder
symptoms.

Toxicity and therapeutic efficacy of compounds can
10 be determined by standard pharmaceutical procedures in
cell cultures or experimental animals, e.g., for
determining the LD50 (the dose lethal to 50% of the
population) and the ED50 (the dose therapeutically
effective in 50% of the population). The dose ratio
15 between toxic and therapeutic effects is the therapeutic
index and it can be expressed as the ratio LD50/ED50.
Compounds which exhibit large therapeutic indices are
preferred. While compounds that exhibit toxic side
effects can be used, care should be taken to design a
20 delivery system that targets such compounds to the site
of affected tissue in order to minimize potential damage
to uninfected cells and, thereby, reduce side effects.

The data obtained from the cell culture assays and
animal studies can be used in formulating a range of
25 dosage for use in humans. The dosage of such compounds
lies preferably within a range of circulating
concentrations that include the ED50 with little or no
toxicity. The dosage can vary within this range depending
upon the dosage form employed and the route of
30 administration utilized. For any compound used in the
methods of the invention, the therapeutically effective
dose can be estimated initially from cell culture assays.
A dose can be formulated in animal models to achieve a
circulating plasma concentration range that includes the
35 IC50 (i.e., the concentration of the test compound which
achieves a half-maximal inhibition of symptoms) as
determined in cell culture. Such information can be used

to more accurately determine useful doses in humans.

- 5 Levels in plasma can be measured, for example, by high performance liquid chromatography.

Pharmaceutical compositions for use in accordance with the present invention can be formulated in conventional manner using one or more physiologically

- 10 acceptable carriers or excipients.

Thus, the compounds and their physiologically acceptable salts and solvents can be formulated for administration by inhalation or insufflation (either through the mouth or the nose) or oral, buccal,

- 15 parenteral or rectal administration.

For oral administration, the pharmaceutical compositions can take the form of, for example, tablets or capsules prepared by conventional means with pharmaceutically acceptable excipients such as binding

- 20 agents (e.g., pregelatinised maize starch, polyvinylpyrrolidone or hydroxypropyl methylcellulose); fillers (e.g., lactose, microcrystalline cellulose or calcium hydrogen phosphate); lubricants (e.g., magnesium stearate, talc or silica); disintegrants (e.g., potato
25 starch or sodium starch glycolate); or wetting agents (e.g., sodium lauryl sulphate). The tablets can be coated by methods well known in the art. Liquid preparations for oral administration can take the form of, for example, solutions, syrups or suspensions, or they can be
30 presented as a dry product for constitution with water or other suitable vehicle before use. Such liquid preparations can be prepared by conventional means with pharmaceutically acceptable additives such as suspending agents (e.g., sorbitol syrup, cellulose derivatives or
35 hydrogenated edible fats); emulsifying agents (e.g., lecithin or acacia); non-aqueous vehicles (e.g., almond oil, oily esters, ethyl alcohol or fractionated vegetable

oils); and preservatives (e.g., methyl or
5 propyl-p-hydroxybenzoates or sorbic acid). The
preparations can also contain buffer salts, flavoring,
coloring and sweetening agents as appropriate.

Preparations for oral administration can be suitably
formulated to give controlled release of the active
10 compound.

For buccal administration the compositions can take
the form of tablets or lozenges formulated in
conventional manner.

For administration by inhalation, the compounds for
15 use according to the present invention are conveniently
delivered in the form of an aerosol spray presentation
from pressurized packs or a nebulizer, with the use of a
suitable propellant, e.g., dichlorodifluoromethane,
trichlorofluoromethane, dichlorotetrafluoroethane, carbon
20 dioxide or other suitable gas. In the case of a
pressurized aerosol the dosage unit can be determined by
providing a valve to deliver a metered amount. Capsules
and cartridges of e.g. gelatin for use in an inhaler or
insufflator can be formulated containing a powder mix of
25 the compound and a suitable powder base such as lactose
or starch.

The compounds can be formulated for parenteral
administration (i.e., intravenous or intramuscular) by
injection, via, for example, bolus injection or
30 continuous infusion. Formulations for injection can be
presented in unit dosage form, e.g., in ampoules or in
multi-dose containers, with an added preservative. The
compositions can take such forms as suspensions,
solutions or emulsions in oily or aqueous vehicles, and
35 can contain formulatory agents such as suspending,
stabilizing and/or dispersing agents. Alternatively, the
active ingredient can be in powder form for constitution

with a suitable vehicle, e.g.; sterile pyrogen-free
 5 water, before use. It is preferred that
 HBMYCNG-expressing cells be introduced into patients via
 intravenous administration.

The compounds can also be formulated in rectal
 compositions such as suppositories or retention enemas,
 10 e.g., containing conventional suppository bases such as
 cocoa butter or other glycerides.

In addition to the formulations described
 previously, the compounds can also be formulated as a
 depot preparation. Such long acting formulations can be
 15 administered by implantation (for example subcutaneously
 or intramuscularly) or by intramuscular injection. Thus,
 for example, the compounds can be formulated with
 suitable polymeric or hydrophobic materials (for example
 as an emulsion in an acceptable oil) or ion exchange
 20 resins, or as sparingly soluble derivatives, for example,
 as a sparingly soluble salt.

The compositions can, if desired, be presented in a
 pack or dispenser device which can contain one or more
 unit dosage forms containing the active ingredient. The
 25 pack can for example comprise metal or plastic foil, such
 as a blister pack. The pack or dispenser device can be
 accompanied by instructions for administration.

6. Example: Identification of Two Novel HBMYCNG Genes and Their Encoded Proteins

30 The section below describes the identification of a
 novel human CNG gene sequence encoding the full-length,
 novel human ion channel, HBMYCNG.

6.1. Cloning of Novel HBMYCNG DNA Sequences

35 In general all routine molecular biology procedures
 followed standard protocols or relied on widely available
 commercial kits and reagents. All sequencing was done

with an ABI 373 automated sequencer using commercial
5 dye-terminator chemistry.

Cyclic nucleotide gated channel sequences from rat,
mouse and chicken were used as sequence probes in a
homology search (gapped BLAST) of public domain expressed
sequence tag (EST) and human genomic databases. The top
10 EST and genomic hits from the BLAST search, (i. e. those
BLAST hits whose Expectation values were less than 0.001
were selected as potential hits and selected for
subsequent analysis) were used as probes in a second
homology search against the non-redundant protein and
15 patent sequence databases. The results of the second
search revealed putative genomic exons which could encode
a novel CNG ion channel, within Bacterial Artificial
Chromosome (BAC), Accession No. AF002992.

The cDNA complete coding sequence of the HBMYCNG
20 gene was cloned as follows. Using the predict full length
sequence The following PCR Primers were designed.

	HuCNG2-s	GCTCTAGATGTACATGGAGGATGACCGAAA	Xba 1 site
25	HuCNG2-1 a	CAGCCAACGCAGTCTGTACTCT	no sites, use nested primer 2
30	HuCNG2-2 a	CGGGATCCGAGGCGGAATCTTGGATGTTT	BamH1 site

Using huCNG2-s and huCNG2-1a, PCR was carried out on
brain first strand cDNA made by standard techniques. To
increase the specificity of the amplification, a 1
35 microliter aliquot was removed after the PCR reaction was
complete and re-amplified using huCNG2-s and huCNG2-2a.
The PCR reaction was passed over a s-400 spun-column

(Amersham Pharmacia Biotech, Piscataway, NJ) to remove
 5 excess PCR primers and DNA was digested with the
 restriction endonucleases Xba I and Bam HI. This reaction
 was extracted with phenol:chloroform and the aqueous
 layer precipitated with 100 % ethanol and 0.3 M Sodium
 Acetate. The precipitated DNA was run on an 0.8% agarose
 10 gel and the DNA band purified using a QIAquick Gel
 extraction kit (Qiagen, Valencia CA). The resulting DNA
 was ligated to pBS-SK digested with Xba I and BamHI
 (Stratagene, La Jolla, CA) and introduced into E. coli
 strain DH10B using standard techniques. Positive clones
 15 were identified by PCR, using the same primers used for
 cloning, and several clones were sequenced using the PCR
 primers as well as with internal primers designed from
 the predicted gene sequence.

20	CNG2-3s	AGAGCCTGCTTCAGTGA	17	Sequencing primer
	CNG2-3a	TCACTGAAGCAGGCTCT	17	Sequencing primer
	CNG2-4s	TTACTGGTCCCACTGA	17	Sequencing primer
25	CNG2-4a	TCAGTGTGGACCAGTAA	17	Sequencing primer
	CNG2-5s	ACGCACAGCTAATATCCGCA	20	Sequencing primer
	CNG2-5a	TGCGGATATTAGCTGTGCGT	20	Sequencing primer

30 The resulting sequence was compared to the predicted
 sequence for completeness.

The DNA sequence for HBMYCNG is depicted in FIG. 1.
 The derived protein, i.e., the full-length amino acid
 35 sequence encoded by the HBMYCNG gene is depicted in FIG.
 2. Analysis of the amino acid sequence of Fig. 2 for the
 detection of transmembrane segments was performed using

the computer program TMPRED and transmembrane prediction
5 information from related proteins. Putative transmembrane
segments are depicted in bold in Fig. 3, while the
predicted ion pore, located between the fifth and sixth
transmembrane, counting from the amino-terminus of the
protein, is underlined.

10 The complete sequence for HBMYCNG can be identified
in a set of sequences from a large genomic fragment
(AF002992) reported as part of the human genome
sequencing project. The complete cDNA nucleotide sequence
encoding the HBMYCNG polypeptide described herein was
15 only partially identified in the annotations to the
AF002992 BAC sequence.

6.2. Calcium Flux Assays Using the HBMYCNG Gene

Ca^{2+} -flux assays are performed to determine the
20 effect on HBMYCNG of various ligands known to affect
cation channel proteins. More specifically, Ca^{2+} uptake is
measured in transiently transfected CHO cells, i.e.,
transfected with the HBMYCNG nucleic acid molecules of
the invention, using the Ca^{2+} -sensitive dye Fluo-4
25 (Molecular Probes) in a Molecular Devices Fluorometric
Imaging Plate Reader (FLIPR). Cells are loaded with the
dye for 30-90 minutes prior to the experiment in the
presence of sulfinpyrazone. Test reagents are added, and
 Ca^{2+} uptake measured over a three minute period.

30 Ca^{2+} -flux assays may also be performed for the
detection and evaluation of compounds that modulate the
activity of G-protein coupled receptors. In such assays,
cells expressing a G-protein coupled receptor of interest
are loaded with the dye for 30-90 minutes prior to the
35 experiment in the presence of sulfinpyrazone. Test
reagents, which include test compounds, which may be
agonists or antagonists of the G-protein coupled receptor

are added, and Ca^{2+} uptake, reflecting the intracellular cyclic nucleotide concentration, is measured over a three minute period. In addition, these same assay techniques can be applied to other cations that enter cells through CNG channels, using appropriate dyes and incubations.

10 6.3. Expression Profile of HBMYCNG

The expression profile of HBMYCNG in various tissues was determined by measuring the relative abundance of HBMYCNG RNA in those tissues using quantitative PCR analyses.

15

Methods

Total RNA from tissues was isolated using the TriZol protocol (Invitrogen, Carlsbad, CA) and quantified by determining absorbance at 260nm. An assessment of the 18S and 28S ribosomal RNA bands was made by denaturing gel electrophoresis to determine RNA integrity.

The specific sequence to be measured was aligned with related genes found in GenBank to identify regions of significant sequence divergence to maximize primer and probe specificity. Gene-specific primers and probes were designed using ABI Primer Express software (Applied Biosystems, Foster City, CA) and used to amplify small amplicons (150 base pairs or less) to maximize the likelihood that the primers would function at 100% efficiency. The primer and probe sequences were searched against Public Genbank databases to ensure target specificity. Primers and probes were obtained from ABI.

For HBMYCNG the primer and probe sequences used were:

35 Forward Primer 5'-TCAGAGAATGGGCCAACAAGA-3'
Reverse Primer 5'-CGAAAACGCTCGAGGAATGA-3'
Probe CAGGCCTAGGTTTCCTCCTCTCGGAAA

5 DNA contamination

To assess the level of contaminating genomic DNA in the RNA, the RNA was divided into 2 aliquots and one half was treated with Rnase-free Dnase (Invitrogen, Carlsbad, CA). RNA from both the Dnase-treated and non-treated samples were then subjected to reverse transcription reactions with (RT+) and without (RT-) the presence of reverse transcriptase. TaqMan™ assays were carried out with the gene-specific primers (see below) and the contribution of genomic DNA to the signal detected was evaluated by comparing the threshold cycles obtained with the RT+/RT- non-Dnase treated RNA to that on the RT+/RT- Dnase treated RNA. For the RNA samples used for the determination of relative expression levels, the amount of signal contributed by genomic DNA in the Dnased RT- RNA was less than 10% of that obtained with Dnased RT+ RNA.

Reverse Transcription reaction and SequenceDetection

25 100ng of Dnase-treated total RNA was annealed to 2.5 mM of the gene-specific reverse primer in the presence of 5.5 mM MgCl₂ by heating the sample to 72°C for 2 min and then cooling to 55° C for 30 min. 1.25 U/ml of MuL_v reverse transcriptase and 500mM of each dNTP were then added to the reaction and the sample was incubated at 37° C for 30 min. The sample was then heated to 90°C for 5 min to denature enzyme.

Quantitative sequence detection was carried out on a ABI PRISM 7700 by adding the following components to the reverse transcribed reaction: forward and reverse primers (each to a concentration of 2.5mM), all four dNTPs (500mM each), buffer and 5U AmpliTaq Gold™. The PCR reaction is

then held at 94°C for 12 min, followed by 40 amplification
5 cycles of 94° C for 15 sec and 60° C for 30 sec.

Data Analysis

The threshold cycle (Ct) of the lowest expressing
tissue (the highest Ct value) was used as the baseline of
expression and all other tissues were expressed as the
10 relative abundance to that tissue by calculating the
difference in Ct value between the baseline and the other
tissues and using it as the exponent in $2^{(\Delta Ct)}$. The
threshold cycles for testis, raphe nucleus, and pineal
gland were 32, 36.5, and 37.5, respectively, indicating
15 that the number of copies of HBMYCNG mRNA in these
samples was very low.

Results

The data obtained indicated that the HBMYCNG gene is
20 expressed only in certain tissues and only at very low
levels in those tissues. More specifically, expression of
the HBMYCNG gene is 250-fold greater in testis, 10-fold
greater in the raphe nucleus of the brain, and 5-fold
greater in the pineal gland than in control tissues.

25

6.4. HBMYCNG Fusion Proteins

Chimeric proteins comprising all or a portion of the
HBMYCNG protein, as depicted in FIG. 2, fused to all or a
portion of a heterologous protein, are provided using
30 recombinant DNA methods and reagents well known in the
art. In specific embodiments, one or more portions of the
HBMYCNG protein are fused to a portion of an
immunoglobulin protein, and, more particularly, to a
portion of a human IgG comprising the hinge, CH2, and CH3
35 regions thereof.

Such portions of the HBMYCNG protein can include,
but are not limited to, one more of the extracellular

- domains of the HBMYCNG protein, comprising,
- 5 approximately, amino acid residues 161 to 173, amino acid residues 237 to 274, and amino acid residues 370 to 453 of SEQ ID No.: 2. In other embodiments, the portion of the HBMYCNG protein incorporated into a fusion includes all or a portion of the amino terminal domain of the
- 10 HBMYCNG protein, comprising, approximately, amino acid residues 1 or 2 to residue 140 SEQ ID No.: 2, or of the carboxy-terminal domain of the HBMYCNG protein, comprising, approximately amino acid residues 474 to 644 of SEQ ID No.: 2.
- 15 DNA encoding the desired portion of the HBMYCNG protein can be isolated by PCR amplification of appropriate sequences, using, for example, cDNA as template, preferably cloned cDNA comprising the nucleotide sequence of SEQ ID NO.: 1, and appropriate
- 20 upstream and downstream primers. The design, synthesis, and use of such primers are well known in the art and will include, as needed or desired, appropriate recognition sequences for one or more restriction enzymes to enable directional, in-frame cloning of a DNA fragment
- 25 encoding a particular portion of the HBMYCNG protein into an expression vector in operable association with appropriate genetic expression and regulatory elements and with a second DNA sequence encoding the protein or portion thereof to which the HBMYCNG protein portion is
- 30 to be fused. Examples of systems useful for the expression of such fusion proteins, in which the HBMYCNG protein portion may be positioned at either the amino-terminus, carboxyl-terminus or within a chimeric fusion protein, are disclosed *supra*.
- 35 HBMYCNG-immunoglobulin C gamma (IgC γ) fusion proteins are prepared as described by Linsley et al., in J. Exp. Med. 173:721-730 (1991), which is hereby incorporated by

reference in its entirety, incorporated by reference
5 herein. DNA encoding amino acid sequences corresponding
to the desired portion of the HBMYCNG protein are joined
to DNA encoding amino acid sequences corresponding to the
hinge, CH2 and CH3 regions of human IgC γ 1. This is
accomplished using PCR amplification to generate DNA
10 fragments encoding appropriate portions of the HBMYCNG
and IgC γ proteins. PCR reactions (0.1 ml final volume)
are run in Taq polymerase buffer (Stratagene, La Jolla,
Calif.), containing 20 μ moles each of dNTP; 50-100 pmoles
of the appropriate primers; template (1 ng plasmid or
15 cDNA synthesized as described by Kawasaki in PCR
Protocols, Academic Press, pp. 21-27 (1990), incorporated
by reference herein); and Taq polymerase (Stratagene).
Reactions are run on a thermocycler (Perkin Elmer Corp.,
Norwalk, Conn.) for 16-30 cycles (a typical cycle consists
20 of steps of 1 min at 94 °C., 1-2 min at 50 °C. and 1-3 min
at 72 °C). Products of the PCR reactions are cleaved with
appropriate restriction endonucleases at sites introduced
in the PCR primers, and then are gel purified.

The 3' portion of the fusion constructs
25 corresponding to human IgC γ 1 sequences is was made by a
coupled reverse transcriptase (from Avian myeloblastosis
virus; Life Sciences Associates, Bayport, N.Y.)--PCR
reaction using RNA from a myeloma cell line producing
human-mouse chimeric mAb L6 (available from Dr. P. Fell
30 and M. Gayle, Bristol-Myers Squibb Company,
Pharmaceutical Research Institute, Seattle, Wash.) as
template. Appropriate upstream and downstream
oligonucleotide, such as those described in U.S. Patent
No. 6,090,914, which is hereby incorporated by reference
35 in its entirety, are used to amplify and isolate the
desired IgC γ coding region.

Reaction products are cleaved with appropriate
5 restriction endonucleases and gel purified. Final
constructs are assembled by ligating the endonucleases
cleaved fragments containing HBMYCNG sequence together
with a cleaved fragment containing IgC γ 1 sequences into
an expression vector such as CDMB, as described in U.S.
10 Patent No. 6,090,917. Ligation products are transformed
into MC1061/p3 *E. coli* cells and colonies are screened
for the appropriate plasmids. Sequences of the resulting
constructs are confirmed by DNA sequencing. In a
preferred embodiment the HBMYCNG portion coding sequence
15 is fused in this manner to DNA encoding amino acids
corresponding to the IgC γ 1 hinge region.

Cell Culture and Transfections

COS (monkey kidney cells) are transfected with
20 expression these chimeric fusion proteins using a
modification of the protocol of Seed and Aruffo (Proc.
Natl. Acad. Sci. 84:3365 (1987)), incorporated by
reference herein. Cells are seeded at 10^6 per 10 cm
diameter culture dish 18-24 h before transfection.
25 Plasmid DNA is added (approximately 15 μ g/dish) in a
volume of 5 mls of serum-free DMEM containing 0.1 mM
chloroquine and 600 μ g/ml DEAE Dextran, and cells are
incubated for 3-3.5 h at 37 °C. Transfected cells are then
briefly treated (approximately 2 min) with 10% dimethyl
30 sulfoxide in PBS and incubated at 37 °C for 16-24 h in
DMEM containing 10% FCS. At 24 h after transfection,
culture medium is removed and replaced with serum-free
DMEM (6 ml/dish). Incubation is continued for 3 days at
37 °C, at which time the spent medium is collected and
35 fresh serum-free medium is added. After an additional 3
days at 37 °C, the spent medium is again collected and
cells are discarded. CHO cells expressing HBMYCNG-IgC γ

fusion proteins are isolated as described by Linsley et al., (1991) supra, as follows: stable transfectants expressing the desired fusion protein are isolated following cotransfection of dihydrofolate reductase-deficient Chinese hamster ovary (dhfr- CHO) cells with a mixture of the appropriate expression plasmid and the selectable marker, pSV2dhfr (Linsley et al., Proc. Natl. Acad. Sci. USA 87:5031 (1990)), incorporated by reference herein. Transfectants are then grown in increasing concentrations of methotrexate to a final level of 1 μ M and were maintained in DMEM supplemented with 10% fetal bovine serum (FBS), 0.2 mM proline and 1 μ M methotrexate. CHO lines expressing high levels of the desired fusion proteins are isolated by multiple rounds of fluorescence-activated cell sorting following indirect immunostaining with an appropriate labeled anti-HBMYCNG mAb.

Purification of Ig Fusion Proteins

The first, second and third collections of spent serum-free culture media from transfected COS cells are used as sources for the purification of Ig fusion proteins. After removal of cellular debris by low speed centrifugation, medium is applied to a column (approximately 200-400 ml medium/ml packed bed volume) of immobilized protein A (Repligen Corp., Cambridge, Mass.) equilibrated with 0.05 M sodium citrate, pH 8.0. After application of the medium, the column is washed with 1 M potassium phosphate, pH 8, and bound protein is eluted with 0.05 M sodium citrate, pH 3. Fractions were collected and immediately neutralized by addition of 1/10 volume of 2 M Tris, pH 8. Fractions containing the peak of A_{280} absorbing material are pooled and dialyzed against PBS before use.

5 6.5 Preparation of Antibodies Directed Against HBMYCNG
Epitopes

Antibodies of the present invention can be prepared by a variety of methods. In one method, purified HBMYCNG
10 antigen or cells expressing purified HBMYCNG antigen are administered to an animal to induce the production of sera containing polyclonal antibodies. In a preferred method, a preparation of HBMYCNG antigen is purified to homogeneity before being administered to an animal to
15 provide polyclonal antisera of greater specific activity. In certain embodiments, soluble portions of the HBMYCNG protein are used as the immunogen for generation of antibodies. Such soluble portions include, but are not limited to extracellular domains of the HBMYCNG protein
20 which comprise, approximately residues 161 to 173, amino acid residues 237 to 274, and amino acid residues 370 to 453 of SEQ ID No.: 2. In other embodiments, a soluble portion of the HBMYCNG protein used as an immunogen may include all or a portion of the amino terminal domain of
25 the HBMYCNG protein, comprising, approximately, amino acid residues 1 or 2 to residue 140 SEQ ID No.: 2, or all or a portion of the carboxy-terminal domain of the HBMYCNG protein, comprising, approximately amino acid residues 474 to 644 of SEQ ID No.: 2. In other
30 embodiments the immunogen administered to the animal may be a chimeric protein or peptide comprising a portion, particularly a soluble portion, of the HBMYCNG protein fused to a protein, polypeptide, or peptide carrier. Such fusions may be constructed by genetic engineering or may
35 be formed by chemical conjugation of the HBMYCNG protein or peptide to a suitable carrier protein or peptide using methods well known in the art.

Monoclonal antibodies specific for the HBMYCNG
 5 protein, or a portion thereof, are prepared using
 hybridoma technology. (Kohler et al., Nature 256:495
 (1975); Kohler et al., Eur. J. Immunol. 6:511 (1976);
 Kohler et al., Eur. J. Immunol. 6:292 (1976); Hammerling
 et al., in: Monoclonal Antibodies and T-Cell Hybridomas,
 10 Elsevier, N.Y., pp. 563-681 (1981)).

An animal, preferably a mouse, is immunized with the
 HBMYCNG protein or a portion thereof and then splenocytes
 of the immunized mice are extracted and fused with a
 suitable myeloma cell line. Any suitable myeloma cell
 15 line may be employed in accordance with the present
 invention; however, it is preferable to employ the parent
 myeloma cell line (SP20), available from the ATCC. After
 fusion, the resulting hybridoma cells are selectively
 maintained in HAT medium, and then cloned by limiting
 20 dilution as described by Wands et al. (Gastroenterology
 80:225-232 (1981). Hybridoma cells obtained through such
 a selection are then assayed to identify clones which
 secrete antibodies capable of binding the HBMYCNG
 polypeptide or portion thereof.

25 For *in vivo* use of antibodies in humans, an antibody
 is "humanized". Such antibodies can be produced using
 genetic constructs derived from hybridoma cells producing
 the monoclonal antibodies described above. Methods for
 producing chimeric and humanized antibodies are known in
 30 the art as disclosed above. (See also, Morrison, Science
 229:1202 (1985); Oi et al., BioTechniques 4:214 (1986);
 Cabilly et al., U.S. Patent No. 4,816,567; Taniguchi et
 al., EP 171496; Morrison et al., EP 173494; Neuberger et
 al., WO 8601533; Robinson et al., WO 8702671; Boulianne
 35 et al., Nature 312:643 (1984); Neuberger et al., Nature
 314:268 (1985).)

Isolation Of Antibody Fragments Directed Against the
HBMYCNG Protein From a Library Of scFvs

5 Naturally occurring V-genes isolated from human peripheral blood lymphocytes (PBLs) are constructed into a library of antibody fragments which contain reactivities against the HBMYCNG protein to which the
 10 donor may or may not have been exposed (see e.g. Marks et al. J. Mol. Bio. 222(3): 581-97 (1991), and U.S. Patent 5,885,793, each of which is incorporated herein by reference in its entirety).

A library of scFvs is constructed from the RNA of
 15 human PBLs as described in PCT publication WO 92/01047, which is hereby incorporated by reference in its entirety. To rescue phage displaying antibody fragments, approximately 10^9 *E. coli* harboring the phagemid are used to inoculate 50 ml of 2xTY containing 1% glucose and 100
 20 μ g/ml of ampicillin (2xTY-AMP-GLU) and grown to an O.D. of 0.8 with shaking. Five ml of this culture are used to innoculate 50 ml of 2xTY-AMP-GLU, 2×10^8 transforming units (TU) of M13 Δ gene III helper phage (PCT publication WO 92/01047) are added and the culture
 25 incubated at 37 °C for 45 minutes without shaking and then at 37 °C for 45 minutes with shaking. The culture is centrifuged at 4000 r.p.m. for 10 min. and the pellet resuspended in 2 liters of 2xTY containing 100 μ g/ml ampicillin and 50 μ g/ml kanamycin and grown overnight.
 30 Phage are prepared as described in PCT publication WO 92/01047.

M13 Δ gene III is prepared as follows: M13 Δ gene III helper phage does not encode gene III protein, hence the phage(mid) displaying antibody fragments have a
 35 greater avidity of binding to antigen. Infectious M13 Δ gene III particles are prepared by growing the helper phage in cells harboring a pUC19 derivative supplying the

- wild type gene III protein during phage morphogenesis.
- 5 The culture is incubated for 1 hour at 37 °C without shaking and then for a further hour at 37 °C with shaking. Cells are collected by centrifugation, resuspended in 300 ml 2xTY broth containing 100 µg ampicillin/ml and 25 µg kanamycin/ml (2xTY-AMP-KAN) and grown overnight, shaking
 - 10 at 37 °C. Phage particles are purified and concentrated from the culture medium by two PEG-precipitations, resuspended in 2 ml PBS and passed through a 0.45 µm filter (Minisart NML; Sartorius) to give a final concentration of approximately 10^{13} transducing units/ml
 - 15 (ampicillin-resistant clones).

Panning of the Library. Immunotubes (Nunc) are coated overnight in PBS with 4 ml of either 100 µg/ml or 10 µg/ml of BMYCNG protein or portion thereof and then blocked with 2% Marvel-PBS for 2 hours at 37 °C and then

- 20 washed 3 times in PBS. Approximately 10^{13} TU of phage is applied to the tube and incubated for 30 minutes at room temperature tumbling on an over and under turntable and then left to stand for another 1.5 hours. Tubes are washed 10 times with PBS 0.1% Tween-20 and 10 times with
- 25 PBS. Phage are eluted by adding 1 ml of 100 mM triethylamine and rotating 15 minutes on an under and over turntable after which the solution is immediately neutralized with 0.5 ml of 1.0M Tris-HCl, pH 7.4. Phage are then used to infect 10 ml of mid-log *E. coli* TG1 by
- 30 incubating eluted phage with bacteria for 30 minutes at 37 °C. The *E. coli* are then plated on TYE plates containing 1% glucose and 100 µg/ml ampicillin. The resulting bacterial library is then rescued with Δ gene III helper phage as described above to prepare phage for
- 35 a subsequent round of selection. This process is then repeated for a total of 4 rounds of affinity purification

with tube-washing increased to 20 times with PBS, 0.1% Tween-20 and 20 times with PBS for rounds 3 and 4.

Eluted phage from the 3rd and 4th rounds of selection are used to infect *E. coli* HB 2151 and soluble scFv is produced (Marks et al. J. Mol. Bio. 222(3): 581-97 (1991)) from single colonies for assay. ELISAs are performed with microtitre plates coated with either 10 pg/ml of HBMYCNG protein or a portion thereof in 50 mM bicarbonate pH 9.6. Clones positive in ELISA are further characterized by PCR fingerprinting (see, e.g., PCT publication WO 92/01047) and then by sequencing.

15

6.5 Method of Creating N- and C-terminal Deletion Mutants Corresponding to the HBMYCNG Polypeptide of the Present Invention.

As described elsewhere herein, the present invention encompasses the creation of N- and C-terminal deletion mutants, in addition to any combination of N- and C-terminal deletions thereof, corresponding to the HBMYCNG polypeptide of the present invention. A number of methods are available to one skilled in the art for creating such mutants. Such methods may include a combination of PCR amplification and gene cloning methodology. Although one of skill in the art of molecular biology, through the use of the teachings provided or referenced herein, and/or otherwise known in the art as standard methods, could readily create each deletion mutant of the present invention, exemplary methods are described below.

Briefly, using the isolated cDNA clone encoding the full-length HBMYCNG polypeptide sequence (as described herein, for example), appropriate primers of about 15-25 nucleotides derived from the desired 5' and 3' positions of SEQ ID NO:1 may be designed to PCR amplify, and subsequently clone, the intended N- and/or C-terminal deletion mutant. Such primers could comprise, for example,

an initiation and stop codon for the 5' and 3' primer,
 5 respectively. Such primers may also comprise restriction
 sites to facilitate cloning of the deletion mutant post
 amplification. Moreover, the primers may comprise
 additional sequences, such as, for example, flag-tag
 sequences, kozac sequences, or other sequences discussed
 10 and/or referenced herein.

For example, in the case of the Y140 to P664 N-
 terminal deletion mutant, the following primers could be
 used to amplify a cDNA fragment corresponding to this
 deletion mutant:

15

5' Prime r	5'-GCAGCA <u>GCGGCCGC</u> TACTACTGCTGGCTATTTGTCATTG-3' (SEQ ID NO:19) NotI
3' Prime r	5'- GCAGCA <u>GTCGAC</u> TGGCTCGTCAGCAGCAGCCAGCTC-3' (SEQ ID NO:20) SalI

20

For example, in the case of the M1 to F475 C-terminal
 deletion mutant, the following primers could be used to
 25 amplify a cDNA fragment corresponding to this deletion
 mutant:

30

5' Prime r	5'- GCAGCA <u>GCGGCCGC</u> ATGACCGAAAAACCAATGGTGTG-3' (SEQ ID NO:21) NotI
3' Prime r	5'- GCAGCA <u>GTCGAC</u> GAAGACCTGAGGACGGAGTTTCAG-3' (SEQ ID NO:22) SalI

Representative PCR amplification conditions are
 35 provided below, although the skilled artisan would
 appreciate that other conditions may be required for
 efficient amplification. A 100 ul PCR reaction mixture may
 be prepared using 10ng of the template DNA (cDNA clone of

HBMYCNG), 200 uM 4dNTPs, 1uM primers, 0.25U Taq DNA
5 polymerase (PE), and standard Taq DNA polymerase buffer.
Typical PCR cycling condition are as follows:

20-25 cycles: 45 sec, 93 degrees
2 min, 50 degrees
2 min, 72 degrees
10 1 cycle: 10 min, 72 degrees

After the final extension step of PCR, 5U Klenow
Fragment may be added and incubated for 15 min at 30
15 degrees.

Upon digestion of the fragment with the NotI and SalI
restriction enzymes, the fragment could be cloned into an
appropriate expression and/or cloning vector which has been
similarly digested (e.g., pSport1, among others). . The
20 skilled artisan would appreciate that other plasmids could
be equally substituted, and may be desirable in certain
circumstances. The digested fragment and vector are then
ligated using a DNA ligase, and then used to transform
competent E.coli cells using methods provided herein and/or
25 otherwise known in the art.

The 5' primer sequence for amplifying any additional
N-terminal deletion mutants may be determined by reference
to the following formula:

$(S+(X * 3))$ to $((S+(X * 3))+25)$, wherein 'S' is equal
30 to the nucleotide position of the initiating start codon of
the HBMYCNG gene (SEQ ID NO:1), and 'X' is equal to the
most N-terminal amino acid of the intended N-terminal
deletion mutant. The first term will provide the start 5'
nucleotide position of the 5' primer, while the second term
will provide the end 3' nucleotide position of the 5'
35 primer corresponding to sense strand of SEQ ID NO:1. Once
the corresponding nucleotide positions of the primer are
determined, the final nucleotide sequence may be created by

the addition of applicable restriction site sequences to the 5' end of the sequence, for example. As referenced herein, the addition of other sequences to the 5' primer may be desired in certain circumstances (e.g., kozac sequences, etc.).

The 3' primer sequence for amplifying any additional N-terminal deletion mutants may be determined by reference to the following formula:

$(S+(X * 3))$ to $((S+(X * 3))-25)$, wherein 'S' is equal to the nucleotide position of the initiating start codon of the HBMYCNG gene (SEQ ID NO:1), and 'X' is equal to the most C-terminal amino acid of the intended N-terminal deletion mutant. The first term will provide the start 5' nucleotide position of the 3' primer, while the second term will provide the end 3' nucleotide position of the 3' primer corresponding to the anti-sense strand of SEQ ID NO:1. Once the corresponding nucleotide positions of the primer are determined, the final nucleotide sequence may be created by the addition of applicable restriction site sequences to the 5' end of the sequence, for example. As referenced herein, the addition of other sequences to the 3' primer may be desired in certain circumstances (e.g., stop codon sequences, etc.). The skilled artisan would appreciate that modifications of the above nucleotide positions may be necessary for optimizing PCR amplification.

The same general formulas provided above may be used in identifying the 5' and 3' primer sequences for amplifying any C-terminal deletion mutant of the present invention. Moreover, the same general formulas provided above may be used in identifying the 5' and 3' primer sequences for amplifying any combination of N-terminal and C-terminal deletion mutant of the present invention. The skilled artisan would appreciate that modifications of the above nucleotide positions may be necessary for optimizing

PCR amplification.

- 5 In preferred embodiments, the following N-terminal HBMYCNG deletion polypeptides are encompassed by the present invention: M1-P664, T2-P664, E3-P664, K4-P664, T5-P664, N6-P664, G7-P664, V8-P664, K9-P664, S10-P664, S11-P664, P12-P664, A13-P664, N14-P664, N15-P664, H16-P664,
- 10 N17-P664, H18-P664, H19-P664, A20-P664, P21-P664, P22-P664, A23-P664, I24-P664, K25-P664, A26-P664, N27-P664, G28-P664, K29-P664, D30-P664, D31-P664, H32-P664, R33-P664, T34-P664, S35-P664, S36-P664, R37-P664, P38-P664, H39-P664, S40-P664, A41-P664, A42-P664, D43-P664, D44-P664, D45-P664, T46-P664,
- 15 S47-P664, S48-P664, E49-P664, L50-P664, Q51-P664, R52-P664, L53-P664, A54-P664, D55-P664, V56-P664, D57-P664, A58-P664, P59-P664, Q60-P664, Q61-P664, G62-P664, R63-P664, S64-P664, G65-P664, F66-P664, R67-P664, R68-P664, I69-P664, V70-P664, R71-P664, L72-P664, V73-P664, G74-P664, I75-P664, I76-P664,
- 20 R77-P664, E78-P664, W79-P664, A80-P664, N81-P664, K82-P664, N83-P664, F84-P664, R85-P664, E86-P664, E87-P664, E88-P664, P89-P664, R90-P664, P91-P664, D92-P664, S93-P664, F94-P664, L95-P664, E96-P664, R97-P664, F98-P664, R99-P664, G100-P664, P101-P664, E102-P664, L103-P664, Q104-P664, T105-P664, V106-P664, T107-P664, T108-P664, Q109-P664, E110-P664, G111-P664, D112-P664, G113-P664, K114-P664, G115-P664, D116-P664, K117-P664, D118-P664, G119-P664, E120-P664, D121-P664, K122-P664, G123-P664, T124-P664, K125-P664, K126-P664, K127-P664, F128-P664, E129-P664, L130-P664, F131-P664, V132-P664, L133-P664, D134-P664, P135-P664, A136-P664, G137-P664, D138-P664, L139-P664, Y140-P664, Y141-P664, C142-P664, W143-P664, L144-P664, F145-P664, V146-P664, I147-P664, A148-P664, M149-P664, P150-P664, V151-P664, L152-P664, Y153-P664, N154-P664, W155-P664, C156-P664, L157-P664, L158-P664, V159-P664, A160-P664, R161-P664, A162-P664, C163-P664, F164-P664, S165-P664, D166-P664, L167-P664, Q168-P664, K169-P664, G170-P664, Y171-P664, Y172-P664, L173-P664, V174-P664, W175-P664,

P664, L176-P664, V177-P664, L178-P664, D179-P664, Y180-
 5 P664, V181-P664, S182-P664, D183-P664, V184-P664, V185-
 P664, Y186-P664, I187-P664, A188-P664, D189-P664, L190-
 P664, F191-P664, I192-P664, R193-P664, L194-P664, R195-
 P664, T196-P664, G197-P664, F198-P664, L199-P664, E200-
 P664, Q201-P664, G202-P664, L203-P664, L204-P664, V205-
 10 P664, K206-P664, D207-P664, T208-P664, K209-P664, K210-
 P664, L211-P664, R212-P664, D213-P664, N214-P664, Y215-
 P664, I216-P664, H217-P664, T218-P664, L219-P664, Q220-
 P664, F221-P664, K222-P664, L223-P664, D224-P664, V225-
 P664, A226-P664, S227-P664, I228-P664, I229-P664, P230-
 15 P664, T231-P664, D232-P664, L233-P664, I234-P664, Y235-
 P664, F236-P664, A237-P664, V238-P664, D239-P664, I240-
 P664, H241-P664, S242-P664, P243-P664, E244-P664, V245-
 P664, R246-P664, F247-P664, N248-P664, R249-P664, L250-
 P664, L251-P664, H252-P664, F253-P664, A254-P664, R255-
 20 P664, M256-P664, F257-P664, E258-P664, F259-P664, F260-
 P664, D261-P664, R262-P664, T263-P664, E264-P664, T265-
 P664, R266-P664, T267-P664, N268-P664, Y269-P664, P270-
 P664, N271-P664, I272-P664, F273-P664, R274-P664, I275-
 P664, S276-P664, N277-P664, L278-P664, V279-P664, L280-
 25 P664, Y281-P664, I282-P664, L283-P664, V284-P664, I285-
 P664, I286-P664, H287-P664, W288-P664, N289-P664, A290-
 P664, C291-P664, I292-P664, Y293-P664, Y294-P664, A295-
 P664, I296-P664, S297-P664, K298-P664, S299-P664, I300-
 P664, G301-P664, F302-P664, G303-P664, V304-P664, D305-
 30 P664, T306-P664, W307-P664, V308-P664, Y309-P664, P310-
 P664, N311-P664, I312-P664, T313-P664, D314-P664, P315-
 P664, E316-P664, Y317-P664, G318-P664, Y319-P664, L320-
 P664, A321-P664, R322-P664, E323-P664, Y324-P664, I325-
 P664, Y326-P664, C327-P664, L328-P664, Y329-P664, W330-
 35 P664, S331-P664, T332-P664, L333-P664, T334-P664, L335-
 P664, T336-P664, T337-P664, I338-P664, G339-P664, E340-
 P664, T341-P664, P342-P664, P343-P664, P344-P664, V345-
 P664, K346-P664, D347-P664, E348-P664, E349-P664, Y350-

P664, L351-P664, F352-P664, V353-P664, I354-P664, F355-
 5 P664, D356-P664, F357-P664, L358-P664, I359-P664, G360-
 P664, V361-P664, L362-P664, I363-P664, F364-P664, A365-
 P664, T366-P664, I367-P664, V368-P664, G369-P664, N370-
 P664, V371-P664, G372-P664, S373-P664, M374-P664, I375-
 P664, S376-P664, N377-P664, M378-P664, N379-P664, A380-
 10 P664, T381-P664, R382-P664, A383-P664, E384-P664, F385-
 P664, Q386-P664, A387-P664, K388-P664, I389-P664, D390-
 P664, A391-P664, V392-P664, K393-P664, H394-P664, Y395-
 P664, M396-P664, Q397-P664, F398-P664, R399-P664, K400-
 P664, V401-P664, S402-P664, K403-P664, G404-P664, M405-
 15 P664, E406-P664, A407-P664, K408-P664, V409-P664, I410-
 P664, R411-P664, W412-P664, F413-P664, D414-P664, Y415-
 P664, L416-P664, W417-P664, T418-P664, N419-P664, K420-
 P664, K421-P664, T422-P664, V423-P664, D424-P664, E425-
 P664, R426-P664, E427-P664, I428-P664, L429-P664, K430-
 20 P664, N431-P664, L432-P664, P433-P664, A434-P664, K435-
 P664, L436-P664, R437-P664, A438-P664, E439-P664, I440-
 P664, A441-P664, T442-P664, N443-P664, V444-P664, H445-
 P664, L446-P664, S447-P664, T448-P664, L449-P664, K450-
 P664, K451-P664, V452-P664, R453-P664, I454-P664, F455-
 25 P664, H456-P664, D457-P664, C458-P664, E459-P664, A460-
 P664, G461-P664, L462-P664, L463-P664, V464-P664, E465-
 P664, L466-P664, V467-P664, L468-P664, K469-P664, L470-
 P664, R471-P664, P472-P664, Q473-P664, V474-P664, F475-
 P664, S476-P664, P477-P664, G478-P664, D479-P664, Y480-
 30 P664, I481-P664, C482-P664, R483-P664, K484-P664, G485-
 P664, D486-P664, I487-P664, G488-P664, K489-P664, E490-
 P664, M491-P664, Y492-P664, I493-P664, I494-P664, K495-
 P664, E496-P664, G497-P664, K498-P664, L499-P664, A500-
 P664, V501-P664, V502-P664, A503-P664, D504-P664, D505-
 35 P664, G506-P664, V507-P664, T508-P664, Q509-P664, Y510-
 P664, A511-P664, L512-P664, L513-P664, S514-P664, A515-
 P664, G516-P664, S517-P664, C518-P664, F519-P664, G520-
 P664, E521-P664, I522-P664, S523-P664, I524-P664, L525-

- 5 P664, N526-P664, I527-P664, K528-P664, G529-P664, S530-P664, K531-P664, M532-P664, G533-P664, N534-P664, R535-P664, R536-P664, T537-P664, A538-P664, N539-P664, I540-P664, R541-P664, S542-P664, L543-P664, G544-P664, Y545-P664, S546-P664, D547-P664, L548-P664, F549-P664, C550-P664, L551-P664, S552-P664, K553-P664, D554-P664, D555-P664, L556-P664, M557-P664, E558-P664, A559-P664, V560-P664, T561-P664, E562-P664, Y563-P664, P564-P664, D565-P664, A566-P664, K567-P664, K568-P664, V569-P664, L570-P664, E571-P664, E572-P664, R573-P664, G574-P664, R575-P664, E576-P664, I577-P664, L578-P664, M579-P664, K580-P664, E581-P664, G582-P664, L583-P664, L584-P664, D585-P664, E586-P664, N587-P664, E588-P664, V589-P664, A590-P664, T591-P664, S592-P664, M593-P664, E594-P664, V595-P664, D596-P664, V597-P664, Q598-P664, E599-P664, K600-P664, L601-P664, G602-P664, Q603-P664, L604-P664, E605-P664, T606-P664, N607-P664, M608-P664, E609-P664, T610-P664, L611-P664, Y612-P664, T613-P664, R614-P664, F615-P664, G616-P664, R617-P664, L618-P664, L619-P664, A620-P664, E621-P664, Y622-P664, T623-P664, G624-P664, A625-P664, Q626-P664, Q627-P664, K628-P664, L629-P664, K630-P664, Q631-P664, R632-P664, I633-P664, T634-P664, V635-P664, L636-P664, E637-P664, T638-P664, K639-P664, M640-P664, K641-P664, Q642-P664, N643-P664, N644-P664, E645-P664, D646-P664, D647-P664, Y648-P664, L649-P664, S650-P664, D651-P664, G652-P664, M653-P664, N654-P664, S655-P664, P656-P664, E657-P664, and/or L658-P664 of SEQ ID NO:2. Polynucleotide sequences encoding these polypeptides are also provided. The present invention also encompasses the use of these N-terminal HBMYCNG deletion polypeptides as immunogenic and/or antigenic epitopes as described elsewhere herein.

35 In preferred embodiments, the following C-terminal HBMYCNG deletion polypeptides are encompassed by the present invention: M1-P664, M1-E663, M1-D662, M1-A661, M1-

A660, M1-A659, M1-L658, M1-E657, M1-P656, M1-S655, M1-N654,
 5 M1-M653, M1-G652, M1-D651, M1-S650, M1-L649, M1-Y648, M1-
 D647, M1-D646, M1-E645, M1-N644, M1-N643, M1-Q642, M1-K641,
 M1-M640, M1-K639, M1-T638, M1-E637, M1-L636, M1-V635, M1-
 T634, M1-I633, M1-R632, M1-Q631, M1-K630, M1-L629, M1-K628,
 M1-Q627, M1-Q626, M1-A625, M1-G624, M1-T623, M1-Y622, M1-
 10 E621, M1-A620, M1-L619, M1-L618, M1-R617, M1-G616, M1-F615,
 M1-R614, M1-T613, M1-Y612, M1-L611, M1-T610, M1-E609, M1-
 M608, M1-N607, M1-T606, M1-E605, M1-L604, M1-Q603, M1-G602,
 M1-L601, M1-K600, M1-E599, M1-Q598, M1-V597, M1-D596, M1-
 V595, M1-E594, M1-M593, M1-S592, M1-T591, M1-A590, M1-V589,
 15 M1-E588, M1-N587, M1-E586, M1-D585, M1-L584, M1-L583, M1-
 G582, M1-E581, M1-K580, M1-M579, M1-L578, M1-I577, M1-E576,
 M1-R575, M1-G574, M1-R573, M1-E572, M1-E571, M1-L570, M1-
 V569, M1-K568, M1-K567, M1-A566, M1-D565, M1-P564, M1-Y563,
 M1-E562, M1-T561, M1-V560, M1-A559, M1-E558, M1-M557, M1-
 20 L556, M1-D555, M1-D554, M1-K553, M1-S552, M1-L551, M1-C550,
 M1-F549, M1-L548, M1-D547, M1-S546, M1-Y545, M1-G544, M1-
 L543, M1-S542, M1-R541, M1-I540, M1-N539, M1-A538, M1-T537,
 M1-R536, M1-R535, M1-N534, M1-G533, M1-M532, M1-K531, M1-
 S530, M1-G529, M1-K528, M1-I527, M1-N526, M1-L525, M1-I524,
 25 M1-S523, M1-I522, M1-E521, M1-G520, M1-F519, M1-C518, M1-
 S517, M1-G516, M1-A515, M1-S514, M1-L513, M1-L512, M1-A511,
 M1-Y510, M1-Q509, M1-T508, M1-V507, M1-G506, M1-D505, M1-
 D504, M1-A503, M1-V502, M1-V501, M1-A500, M1-L499, M1-K498,
 M1-G497, M1-E496, M1-K495, M1-I494, M1-I493, M1-Y492, M1-
 30 M491, M1-E490, M1-K489, M1-G488, M1-I487, M1-D486, M1-G485,
 M1-K484, M1-R483, M1-C482, M1-I481, M1-Y480, M1-D479, M1-
 G478, M1-P477, M1-S476, M1-F475, M1-V474, M1-Q473, M1-P472,
 M1-R471, M1-L470, M1-K469, M1-L468, M1-V467, M1-L466, M1-
 E465, M1-V464, M1-L463, M1-L462, M1-G461, M1-A460, M1-E459,
 M1-C458, M1-D457, M1-H456, M1-F455, M1-I454, M1-R453, M1-
 35 V452, M1-K451, M1-K450, M1-L449, M1-T448, M1-S447, M1-L446,
 M1-H445, M1-V444, M1-N443, M1-T442, M1-A441, M1-I440, M1-
 E439, M1-A438, M1-R437, M1-L436, M1-K435, M1-A434, M1-P433,

M1-L432, M1-N431, M1-K430, M1-L429, M1-I428, M1-E427, M1-
 5 R426, M1-E425, M1-D424, M1-V423, M1-T422, M1-K421, M1-K420,
 M1-N419, M1-T418, M1-W417, M1-L416, M1-Y415, M1-D414, M1-
 F413, M1-W412, M1-R411, M1-I410, M1-V409, M1-K408, M1-A407,
 M1-E406, M1-M405, M1-G404, M1-K403, M1-S402, M1-V401, M1-
 K400, M1-R399, M1-F398, M1-Q397, M1-M396, M1-Y395, M1-H394,
 10 M1-K393, M1-V392, M1-A391, M1-D390, M1-I389, M1-K388, M1-
 A387, M1-Q386, M1-F385, M1-E384, M1-A383, M1-R382, M1-T381,
 M1-A380, M1-N379, M1-M378, M1-N377, M1-S376, M1-I375, M1-
 M374, M1-S373, M1-G372, M1-V371, M1-N370, M1-G369, M1-V368,
 M1-I367, M1-T366, M1-A365, M1-F364, M1-I363, M1-L362, M1-
 15 V361, M1-G360, M1-I359, M1-L358, M1-F357, M1-D356, M1-F355,
 M1-I354, M1-V353, M1-F352, M1-L351, M1-Y350, M1-E349, M1-
 E348, M1-D347, M1-K346, M1-V345, M1-P344, M1-P343, M1-P342,
 M1-T341, M1-E340, M1-G339, M1-I338, M1-T337, M1-T336, M1-
 L335, M1-T334, M1-L333, M1-T332, M1-S331, M1-W330, M1-Y329,
 20 M1-L328, M1-C327, M1-Y326, M1-I325, M1-Y324, M1-E323, M1-
 R322, M1-A321, M1-L320, M1-Y319, M1-G318, M1-Y317, M1-E316,
 M1-P315, M1-D314, M1-T313, M1-I312, M1-N311, M1-P310, M1-
 Y309, M1-V308, M1-W307, M1-T306, M1-D305, M1-V304, M1-G303,
 M1-F302, M1-G301, M1-I300, M1-S299, M1-K298, M1-S297, M1-
 25 I296, M1-A295, M1-Y294, M1-Y293, M1-I292, M1-C291, M1-A290,
 M1-N289, M1-W288, M1-H287, M1-I286, M1-I285, M1-V284, M1-
 L283, M1-I282, M1-Y281, M1-L280, M1-V279, M1-L278, M1-N277,
 M1-S276, M1-I275, M1-R274, M1-F273, M1-I272, M1-N271, M1-
 P270, M1-Y269, M1-N268, M1-T267, M1-R266, M1-T265, M1-E264,
 30 M1-T263, M1-R262, M1-D261, M1-F260, M1-F259, M1-E258, M1-
 F257, M1-M256, M1-R255, M1-A254, M1-F253, M1-H252, M1-L251,
 M1-L250, M1-R249, M1-N248, M1-F247, M1-R246, M1-V245, M1-
 E244, M1-P243, M1-S242, M1-H241, M1-I240, M1-D239, M1-V238,
 M1-A237, M1-F236, M1-Y235, M1-I234, M1-L233, M1-D232, M1-
 T231, M1-P230, M1-I229, M1-I228, M1-S227, M1-A226, M1-V225,
 35 M1-D224, M1-L223, M1-K222, M1-F221, M1-Q220, M1-L219, M1-
 T218, M1-H217, M1-I216, M1-Y215, M1-N214, M1-D213, M1-R212,
 M1-L211, M1-K210, M1-K209, M1-T208, M1-D207, M1-K206, M1-

V205, M1-L204, M1-L203, M1-G202, M1-Q201, M1-E200, M1-L199,
 5 M1-F198, M1-G197, M1-T196, M1-R195, M1-L194, M1-R193, M1-
 I192, M1-F191, M1-L190, M1-D189, M1-A188, M1-I187, M1-Y186,
 M1-V185, M1-V184, M1-D183, M1-S182, M1-V181, M1-Y180, M1-
 D179, M1-L178, M1-V177, M1-L176, M1-W175, M1-V174, M1-L173,
 M1-Y172, M1-Y171, M1-G170, M1-K169, M1-Q168, M1-L167, M1-
 10 D166, M1-S165, M1-F164, M1-C163, M1-A162, M1-R161, M1-A160,
 M1-V159, M1-L158, M1-L157, M1-C156, M1-W155, M1-N154, M1-
 Y153, M1-L152, M1-V151, M1-P150, M1-M149, M1-A148, M1-I147,
 M1-V146, M1-F145, M1-L144, M1-W143, M1-C142, M1-Y141, M1-
 Y140, M1-L139, M1-D138, M1-G137, M1-A136, M1-P135, M1-D134,
 15 M1-L133, M1-V132, M1-F131, M1-L130, M1-E129, M1-F128, M1-
 K127, M1-K126, M1-K125, M1-T124, M1-G123, M1-K122, M1-D121,
 M1-E120, M1-G119, M1-D118, M1-K117, M1-D116, M1-G115, M1-
 K114, M1-G113, M1-D112, M1-G111, M1-E110, M1-Q109, M1-T108,
 M1-T107, M1-V106, M1-T105, M1-Q104, M1-L103, M1-E102, M1-
 20 P101, M1-G100, M1-R99, M1-F98, M1-R97, M1-E96, M1-L95, M1-
 F94, M1-S93, M1-D92, M1-P91, M1-R90, M1-P89, M1-E88, M1-
 E87, M1-E86, M1-R85, M1-F84, M1-N83, M1-K82, M1-N81, M1-
 A80, M1-W79, M1-E78, M1-R77, M1-I76, M1-I75, M1-G74, M1-
 V73, M1-L72, M1-R71, M1-V70, M1-I69, M1-R68, M1-R67, M1-
 25 F66, M1-G65, M1-S64, M1-R63, M1-G62, M1-Q61, M1-Q60, M1-
 P59, M1-A58, M1-D57, M1-V56, M1-D55, M1-A54, M1-L53, M1-
 R52, M1-Q51, M1-L50, M1-E49, M1-S48, M1-S47, M1-T46, M1-
 D45, M1-D44, M1-D43, M1-A42, M1-A41, M1-S40, M1-H39, M1-
 P38, M1-R37, M1-S36, M1-S35, M1-T34, M1-R33, M1-H32, M1-
 30 D31, M1-D30, M1-K29, M1-G28, M1-N27, M1-A26, M1-K25, M1-
 I24, M1-A23, M1-P22, M1-P21, M1-A20, M1-H19, M1-H18, M1-
 N17, M1-H16, M1-N15, M1-N14, M1-A13, M1-P12, M1-S11, M1-
 S10, M1-K9, M1-V8, and/or M1-G7 of SEQ ID NO:2.
 Polynucleotide sequences encoding these polypeptides are
 also provided. The present invention also encompasses the
 35 use of these C-terminal HBMYCNG deletion polypeptides as
 immunogenic and/or antigenic epitopes as described
 elsewhere herein.

The present invention also encompasses the the same N-
5 and/or C-terminal deletion mutants for the varant HBMYCNG
polypeptide depicted in FIG. 6 (SEQ ID NO:24) with the
appropriate amino acid and encoding nucleic acid
substitutions. Methods of substituting such sequences are
known in the art.

10

6.6 Method Of Enhancing The Biological Activity/Functional
Characteristics Of Invention Through Molecular Evolution.

Although many of the most biologically active proteins
known are highly effective for their specified function in
15 an organism, they often possess characteristics that make
them undesirable for transgenic, therapeutic,
pharmaceutical, and/or industrial applications. Among these
traits, a short physiological half-life is the most
prominent problem, and is present either at the level of
20 the protein, or the level of the proteins mRNA. The ability
to extend the half-life, for example, would be particularly
important for a proteins use in gene therapy, transgenic
animal production, the bioprocess production and
purification of the protein, and use of the protein as a
25 chemical modulator among others. Therefore, there is a need
to identify novel variants of isolated proteins possessing
characteristics which enhance their application as a
therapeutic for treating diseases of animal origin, in
addition to the proteins applicability to common industrial
30 and pharmaceutical applications.

Thus, one aspect of the present invention relates to
the ability to enhance specific characteristics of
invention through directed molecular evolution. Such an
enhancement may, in a non-limiting example, benefit the
inventions utility as an essential component in a kit, the
35 inventions physical attributes such as its solubility,
structure, or codon optimization, the inventions specific
biological activity, including any associated enzymatic

activity, the proteins enzyme kinetics, the proteins K_i ,
5 K_{cat} , K_m , V_{max} , K_d , protein-protein activity, protein-DNA
binding activity, antagonist/inhibitory activity (including
direct or indirect interaction), agonist activity
(including direct or indirect interaction), the proteins
antigenicity (e.g., where it would be desirable to either
10 increase or decrease the antigenic potential of the
protein), the immunogenicity of the protein, the ability of
the protein to form dimers, trimers, or multimers with
either itself or other proteins, the antigenic efficacy of
the invention, including its subsequent use a preventative
15 treatment for disease or disease states, or as an effector
for targeting diseased genes. Moreover, the ability to
enhance specific characteristics of a protein may also be
applicable to changing the characterized activity of an
enzyme to an activity completely unrelated to its initially
20 characterized activity. Other desirable enhancements of the
invention would be specific to each individual protein, and
would thus be well known in the art and contemplated by the
present invention.

For example, an engineered ion channel protein may be
25 constitutively active upon binding of its cognate ligand.
Alternatively, an engineered ion channel protein may be
constitutively active in the absence of ligand binding. In
yet another example, an engineered ion channel protein may
be capable of being activated with less than all of the
regulatory factors and/or conditions typically required for
30 ion channel protein activation (e.g., ion flux, ligand
binding, phosphorylation, conformational changes, etc.).
Such ion channel protein would be useful in screens to
identify ion channel protein modulators, among other uses
described herein.

35 Directed evolution is comprised of several steps. The
first step is to establish a library of variants for the
gene or protein of interest. The most important step is to

then select for those variants that entail the activity you
5 wish to identify. The design of the screen is essential
since your screen should be selective enough to eliminate
non-useful variants, but not so stringent as to eliminate
all variants. The last step is then to repeat the above
steps using the best variant from the previous screen. Each
10 successive cycle, can then be tailored as necessary, such
as increasing the stringency of the screen, for example.

Over the years, there have been a number of methods
developed to introduce mutations into macromolecules. Some
of these methods include, random mutagenesis, "error-prone"
15 PCR, chemical mutagenesis, site-directed mutagenesis, and
other methods well known in the art (for a comprehensive
listing of current mutagenesis methods, see Maniatis,
Molecular Cloning: A Laboratory Manual, Cold Spring Harbor
Press, Cold Spring, NY (1982)). Typically, such methods
20 have been used, for example, as tools for identifying the
core functional region(s) of a protein or the function of
specific domains of a protein (if a multi-domain protein).
However, such methods have more recently been applied to
the identification of macromolecule variants with specific
25 or enhanced characteristics.

Random mutagenesis has been the most widely recognized
method to date. Typically, this has been carried out either
through the use of "error-prone" PCR (as described in
Moore, J., et al, Nature Biotechnology 14:458, (1996), or
30 through the application of randomized synthetic
oligonucleotides corresponding to specific regions of
interest (as described by Derbyshire, K.M. et al, Gene,
46:145-152, (1986), and Hill, DE, et al, Methods Enzymol.,
55:559-568, (1987). Both approaches have limits to the
level of mutagenesis that can be obtained. However, either
35 approach enables the investigator to effectively control
the rate of mutagenesis. This is particularly important
considering the fact that mutations beneficial to the

activity of the enzyme are fairly rare. In fact, using too
5 high a level of mutagenesis may counter or inhibit the
desired benefit of a useful mutation.

While both of the aforementioned methods are effective
for creating randomized pools of macromolecule variants, a
third method, termed "DNA Shuffling", or "sexual PCR" (WPC,
10 Stemmer, PNAS, 91:10747, (1994)) has recently been
elucidated. DNA shuffling has also been referred to as
"directed molecular evolution", "exon-shuffling", "directed
enzyme evolution", "in vitro evolution", and "artificial
evolution". Such reference terms are known in the art and
15 are encompassed by the invention. This new, preferred,
method apparently overcomes the limitations of the previous
methods in that it not only propagates positive traits, but
simultaneously eliminates negative traits in the resulting
progeny.

20 DNA shuffling accomplishes this task by combining the
principal of in vitro recombination, along with the method
of "error-prone" PCR. In effect, you begin with a randomly
digested pool of small fragments of your gene, created by
Dnase I digestion, and then introduce said random fragments
25 into an "error-prone" PCR assembly reaction. During the PCR
reaction, the randomly sized DNA fragments not only
hybridize to their cognate strand, but also may hybridize
to other DNA fragments corresponding to different regions
of the polynucleotide of interest - regions not typically
30 accessible via hybridization of the entire polynucleotide.
Moreover, since the PCR assembly reaction utilizes "error-
prone" PCR reaction conditions, random mutations are
introduced during the DNA synthesis step of the PCR
reaction for all of the fragments -further diversifying the
35 potential hybridation sites during the annealing step of
the reaction.

A variety of reaction conditions could be utilized to
carry-out the DNA shuffling reaction. However, specific

reaction conditions for DNA shuffling are provided, for
5 example, in PNAS, 91:10747, (1994). Briefly:

Prepare the DNA substrate to be subjected to the DNA
shuffling reaction. Preparation may be in the form of
simply purifying the DNA from contaminating cellular
material, chemicals, buffers, oligonucleotide primers,
10 deoxynucleotides, RNAs, etc., and may entail the use of DNA
purification kits as those provided by Qiagen, Inc., or by
the Promega, Corp., for example.

Once the DNA substrate has been purified, it would be
subjected to Dnase I digestion. About 2-4ug of the DNA
15 substrate(s) would be digested with .0015 units of Dnase I
(Sigma) per ul in 100ul of 50mM Tris-HCL, pH 7.4/1mM MgCl₂
for 10-20 min. at room temperature. The resulting fragments
of 10-50bp could then be purified by running them through
a 2% low-melting point agarose gel by electrophoresis onto
20 DE81 ion-exchange paper (Whatman) or could be purified
using Microcon concentrators (Amicon) of the appropriate
molecular weight cutoff, or could use oligonucleotide
purification columns (Qiagen), in addition to other methods
known in the art. If using DE81 ion-exchange paper, the 10-
25 50bp fragments could be eluted from said paper using 1M
NaCl, followed by ethanol precipitation.

The resulting purified fragments would then be
subjected to a PCR assembly reaction by re-suspension in a
PCR mixture containing: 2mM of each dNTP, 2.2mM MgCl₂, 50
30 mM KCl, 10mM Tris•HCL, pH 9.0, and 0.1% Triton X-100, at a
final fragment concentration of 10-30ng/ul. No primers are
added at this point. Taq DNA polymerase (Promega) would be
used at 2.5 units per 100ul of reaction mixture. A PCR
program of 94 C for 60s; 94 C for 30s, 50-55 C for 30s, and
35 72 C for 30s using 30-45 cycles, followed by 72 C for 5min
using an MJ Research (Cambridge, MA) PTC-150 thermocycler.
After the assembly reaction is completed, a 1:40 dilution
of the resulting primerless product would then be

introduced into a PCR mixture (using the same buffer
5 mixture used for the assembly reaction) containing 0.8um of
each primer and subjecting this mixture to 15 cycles of PCR
(using 94 C for 30s, 50 C for 30s, and 72 C for 30s). The
referred primers would be primers corresponding to the
nucleic acid sequences of the polynucleotide(s) utilized in
10 the shuffling reaction. Said primers could consist of
modified nucleic acid base pairs using methods known in the
art and referred to else where herein, or could contain
additional sequences (i.e., for adding restriction sites,
mutating specific base-pairs, etc.).

15 The resulting shuffled, assembled, and amplified
product can be purified using methods well known in the art
(e.g., Qiagen PCR purification kits) and then subsequently
cloned using appropriate restriction enzymes.

Although a number of variations of DNA shuffling have
20 been published to date, such variations would be obvious to
the skilled artisan and are encompassed by the invention.
The DNA shuffling method can also be tailored to the
desired level of mutagenesis using the methods described by
Zhao, et al. (Nucl Acid Res., 25(6):1307-1308, (1997)).

25 As described above, once the randomized pool has been
created, it can then be subjected to a specific screen to
identify the variant possessing the desired
characteristic(s). Once the variant has been identified,
DNA corresponding to the variant could then be used as the
DNA substrate for initiating another round of DNA
30 shuffling. This cycle of shuffling, selecting the optimized
variant of interest, and then re-shuffling, can be repeated
until the ultimate variant is obtained. Examples of model
screens applied to identify variants created using DNA
shuffling technology may be found in the following
35 publications: J. C., Moore, et al., J. Mol. Biol., 272:336-
347, (1997), F.R., Cross, et al., Mol. Cell. Biol.,
18:2923-2931, (1998), and A. Cramer, et al., Nat.

Biotech., 15:436-438, (1997).

5 DNA shuffling has several advantages. First, it makes
use of beneficial mutations. When combined with screening,
DNA shuffling allows the discovery of the best mutational
combinations and does not assume that the best combination
contains all the mutations in a population. Secondly,
10 recombination occurs simultaneously with point mutagenesis.
An effect of forcing DNA polymerase to synthesize full-
length genes from the small fragment DNA pool is a
background mutagenesis rate. In combination with a
stringent selection method, enzymatic activity has been
15 evolved up to 16000 fold increase over the wild-type form
of the enzyme. In essence, the background mutagenesis
yielded the genetic variability on which recombination
acted to enhance the activity.

A third feature of recombination is that it can be
20 used to remove deleterious mutations. As discussed above,
during the process of the randomization, for every one
beneficial mutation, there may be at least one or more
neutral or inhibitory mutations. Such mutations can be
removed by including in the assembly reaction an excess of
25 the wild-type random-size fragments, in addition to the
random-size fragments of the selected mutant from the
previous selection. During the next selection, some of the
most active variants of the polynucleotide/
polypeptide/enzyme, should have lost the inhibitory
30 mutations.

Finally, recombination enables parallel processing.
This represents a significant advantage since there are
likely multiple characteristics that would make a protein
more desirable (e.g. solubility, activity, etc.). Since it
35 is increasingly difficult to screen for more than one
desirable trait at a time, other methods of molecular
evolution tend to be inhibitory. However, using
recombination, it would be possible to combine the

randomized fragments of the best representative variants
5 for the various traits, and then select for multiple
properties at once.

DNA shuffling can also be applied to the
polynucleotides and polypeptides of the present invention
to decrease their immunogenicity in a specified host,
10 particularly if the polynucleotides and polypeptides
provide a therapeutic use. For example, a particular
variant of the present invention may be created and
isolated using DNA shuffling technology. Such a variant may
have all of the desired characteristics, though may be
15 highly immunogenic in a host due to its novel intrinsic
structure. Specifically, the desired characteristic may
cause the polypeptide to have a non-native structure which
could no longer be recognized as a "self" molecule, but
rather as a "foreign", and thus activate a host immune
20 response directed against the novel variant. Such a
limitation can be overcome, for example, by including a
copy of the gene sequence for a xenobiotic ortholog of the
native protein in with the gene sequence of the novel
variant gene in one or more cycles of DNA shuffling. The
25 molar ratio of the ortholog and novel variant DNAs could be
varied accordingly. Ideally, the resulting hybrid variant
identified would contain at least some of the coding
sequence which enabled the xenobiotic protein to evade the
host immune system, and additionally, the coding sequence
30 of the original novel variant that provided the desired
characteristics.

Likewise, the invention encompasses the application of
DNA shuffling technology to the evolution of
polynucleotides and polypeptides of the invention, wherein
one or more cycles of DNA shuffling include, in addition to
35 the gene template DNA, oligonucleotides coding for known
allelic sequences, optimized codon sequences, known variant
sequences, known polynucleotide polymorphism sequences,

known ortholog sequences, known homolog sequences,
 5 additional homologous sequences, additional non-homologous
 sequences, sequences from another species, and any number
 and combination of the above.

In addition to the described methods above, there are
 a number of related methods that may also be applicable, or
 10 desirable in certain cases. Representative among these are
 the methods discussed in PCT applications WO 98/31700, and
 WO 98/32845, which are hereby incorporated by reference.
 Furthermore, related methods can also be applied to the
 polynucleotide sequences of the present invention in order
 15 to evolve invention for creating ideal variants for use in
 gene therapy, protein engineering, evolution of whole cells
 containing the variant, or in the evolution of entire
 enzyme pathways containing polynucleotides of the invention
 as described in PCT applications WO 98/13485, WO 98/13487,
 20 WO 98/27230, WO 98/31837, and Crameri, A., et al., Nat.
 Biotech., 15:436-438, (1997), respectively.

Additional methods of applying "DNA Shuffling"
 technology to the polynucleotides and polypeptides of the
 present invention, including their proposed applications,
 25 may be found in US Patent No. 5,605,793; PCT Application
 No. WO 95/22625; PCT Application No. WO 97/20078; PCT
 Application No. WO 97/35966; and PCT Application No. WO
 98/42832; PCT Application No. The forgoing are hereby
 incorporated in their entirety herein for all purposes.

30 7. DEPOSIT OF MICROORGANISMS

The following microorganisms were deposited with the
 American Type Culture Collection (ATCC), 10801 University
 Blvd., Manassas, Virginia 20110 on _____ and
 assigned the following numbers:

35

Microorganism
 HBMYCNG-pcDNA

ATCC Deposit No.

5 The present invention is not to be limited in scope
by the specific embodiments described herein, which are
intended as single illustrations of individual aspects of
the invention, and functionally equivalent methods and
components are within the scope of the invention. Indeed,
10 various modifications of the invention, in addition to
those shown and described herein will become apparent to
those skilled in the art from the foregoing description
and accompanying drawings. Such modifications are
intended to fall within the scope of the appended claims.
15 Various publications are cited herein, the
disclosures of which are incorporated by reference in
their entireties.

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